

ANALYSIS OF A NOVEL PHENAZINE
ANTIBIOTIC GENE CLUSTER IN *ERWINIA*
HERBICOLA EH1087: A BIOLOGICAL
CONTROL AGENT FOR FIREBLIGHT

Stephen Russel Giddens

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List of Abbreviations

1-A	minimal 1-A medium
aa	amino acid
AGA	D-alanylgriseoluteic acid
AP1	antibiotic precursor one
bp	base pair
°C	degrees Celsius
Da	Dalton
dH ₂ O	double distilled water
dsDNA	double stranded DNA
ECL	enhanced chemiluminescence
EhehpX	Eh1087 mutant interrupted in <i>ehp</i> gene X
<i>ehp</i>	<i>Erwinia herbicola</i> phenazine gene
g	gravity
GGA	glycylgriseoluteic acid
h	hour
HVGA	hydroxyvalylgriseoluteic acid
kbp	kilobase pair
kDa	kilodalton
LB	Luria Bertani medium
mA	milliamps
MIC	minimal inhibitory concentration
min	minute
MLC	minimal lethal concentration
OD _{xxx}	optical density at wavelength xxx nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
<i>phz</i>	genes involved in phenazine biosynthesis in <i>Pseudomonas</i> spp.
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
UV	ultraviolet
VGA	valylgriseoluteic acid

University of Canterbury

Abstract

ANALYSIS OF A NOVEL PHENAZINE ANTIBIOTIC GENE
CLUSTER IN *ERWINIA HERBICOLA* EH1087: A
BIOLOGICAL CONTROL AGENT FOR FIREBLIGHT

Stephen Giddens

The present study advances genetic, molecular biology, and biochemical analyses of antibiotic production by *Erwinia herbicola* (syn. *Pantoea agglomerans*) strain Eh1087, a candidate for biological control of the Fire Blight pathogen *Erwinia amylovora*. Three research areas were investigated concurrently: i) the role of antibiosis in biological control by Eh1087, ii) the genetic basis of antibiotic production, and iii) the nature of the antibiotic. Antibiotic production was shown to be an important biological control mechanism since removal of an antibiotic synthesis gene cluster in Eh1087 significantly decreased the ability of this bacterium to suppress the colonization of apple blossom stigmas by *E. amylovora* strain Ea8862.

A region of DNA sufficient for antibiotic production and resistance was identified and characterized by molecular genetic analyses. Evidence suggesting that this DNA region did not originate in Eh1087 is presented. The region was found to contain a cluster of 16 genes designated *ehp* (*Erwinia herbicola* phenazine) using a combination of DNA sequence analyses, minicell protein analyses, and the correlation of mutation with the production of coloured AGA intermediates by many *ehp* mutants. The function and regulation of the genes within the cluster were determined by the use of classical syntrophic and DNA-complementation experiments. Each biosynthetic gene was assigned to one of four Groups according to the function of their products in antibiotic biosynthesis, while a single gene was shown to provide antibiotic resistance. A pathway for antibiotic biosynthesis is proposed that involves intracellular synthesis of a phenazine nucleus, modification and translocation of this nucleus to the periplasm, and finally the activation and release of antibiotic from the producing cell.

Purification and analysis of the Eh1087 antibiotic demonstrated that the *ehp* genes confer on Eh1087 the ability to produce the phenazine antibiotic D-alanylgriseoluteic acid (AGA). This is the first example of the isolation of a phenazine antibiotic from a species of *Erwinia* (or *Pantoea*), and only the second isolation of AGA as a natural product. The characterization of various AGA properties led to predictions about its mode of action.

Chapter 1. INTRODUCTION

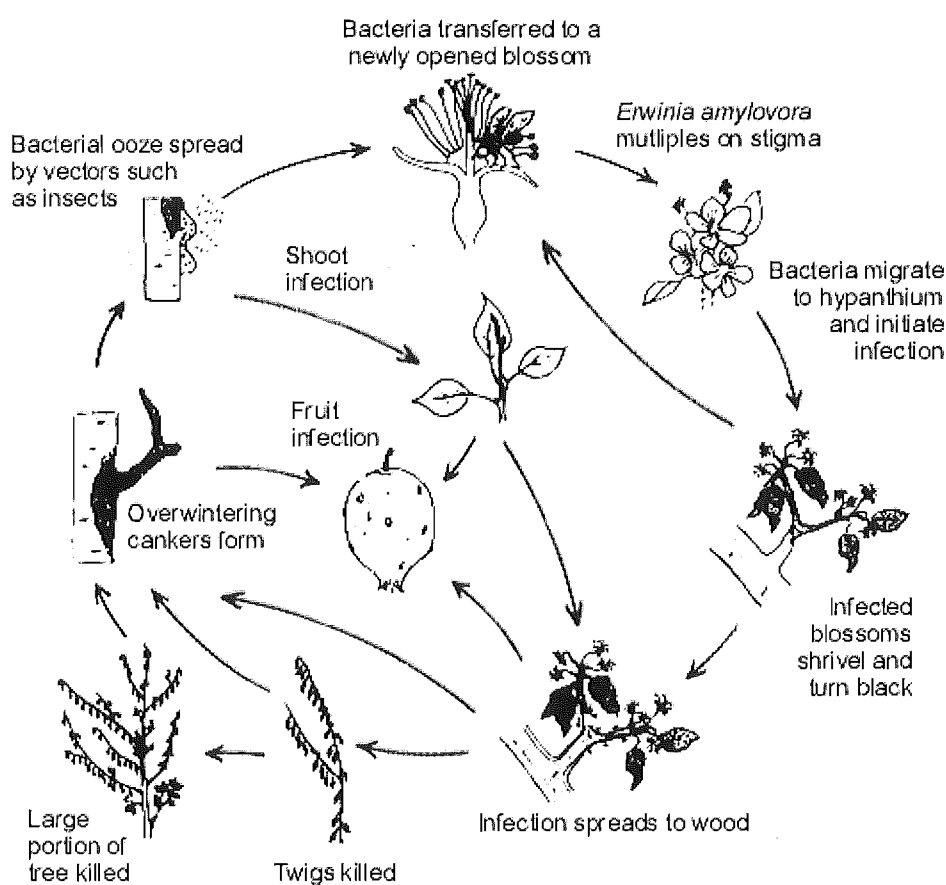
In many parts of the world, including the US, Europe, The Middle East, and New Zealand, apple and pear growers face a constant battle against a disease called Fire Blight (Bonn and van der Zwet, 2000). Fire Blight is a necrotic disease so-named because the tissues of badly infected plants become blackened, as if ravaged by fire. The infectious agent responsible for Fire Blight is the bacterium *Erwinia amylovora* (originally *Bacillus amylovora*), which was the first bacterium to be identified as a plant pathogen (Burrill, 1883). Over the intervening years the disease cycle of *E. amylovora* has been well established (Fig. 1.1). Recent studies have shed light both on the likely sites of infection (blossoms and shoots) and on *E. amylovora*-specific factors involved in the disease process (recently reviewed by Eastgate, 2000). Much of this research has been motivated by difficulties in the control of Fire Blight. These difficulties have become more troublesome with the use of most modern apple and pear cultivars, which are particularly susceptible to Fire Blight as they were developed for marketability and not for disease-resistance (Johnson and Stockwell, 1998).

Fire Blight

An understanding of the disease cycle of *E. amylovora* is essential for the development of methods to control Fire Blight. *E. amylovora* over-winters inside a host plant as a concentrated area of infection termed a 'canker' (Fig. 1.1). Once spring arrives, vectors such as insects or rain can transfer the bacterium to newly opened blossoms (Johnson et al., 1993). This marks the start of an epiphytic phase during which *E. amylovora* multiplies to $10^6 - 10^7$ CFU.blossom⁻¹ in the moist, nutrient rich environment of the stigma (Gibbins, 1978; Johnson and Stockwell, 1998; Wilson et al., 1992; Wilson and Lindow, 1993). The high epiphytic population of *E. amylovora* on the stigma can provide inocula for transfer to new blossoms, enabling the bacterium to rapidly infect large areas of an orchard (Johnson et al., 1993). *E. amylovora* does not survive well on flower parts other than the stigma (Rundle and Beer, 1987; Thomson, 1986), however it has to migrate from the stigma down the style to the hypanthium (nectaries) to infect plant tissue and initiate the blossom blight phase of Fire Blight. It is therefore likely that an epiphytic phase results in a population of *E. amylovora* sufficient to overwhelm the natural plant

defenses at the hypanthium (Thomson, 1986). Since infections usually follow rainstorms or heavy dew, the moisture from such events is thought to transfer *E. amylovora* from the stigma to the hypanthium and may also act to dilute out natural defenses, such as the high osmolarity of nectar, at the hypanthium (Johnson and Stockwell, 1998; Stockwell et al., 1999; Thomson, 1986). After infecting a plant, *E. amylovora* can rapidly spread throughout the host and eventually cause systemic disease, sometimes resulting in the death of a tree in a single season (Eastgate, 2000; Vanneste, 1996).

Figure 1.1. The Fire Blight disease cycle



In the last two decades or so, a number of factors required by *E. amylovora* to cause Fire Blight has been elucidated. As recently reviewed by Eastgate (2000), *E. amylovora* has distinct

features that enable it to survive within the plant, and to cause disease. Survival factors include siderophores to scavenge iron in the low iron environment of the plant and the ability to metabolize sugars such as sucrose and sorbitol, a major transport sugar in pear. Disease factors have largely been uncovered by the analysis of *E. amylovora* mutants that lack the ability to cause disease. For instance, mutants unable to produce an extracellular polysaccharide named amylovoran were found to be unencapsulated and avirulent (Roberts and Coleman, 1991). The mutations responsible for the avirulence phenotype were located adjacent to a ca. 25 kbp region of DNA required for hypersensitive response and pathogenicity (*hrp* genes) (Bogdanove et al., 1998). The product of *hrpN*, harpin is an intracellular protein that elicits a hypersensitive response in tobacco leaves and is a Fire Blight pathogenicity factor (Wei et al., 1992). Expression of the *hrp* genes is influenced by carbon source, pH, temperature, and ammonium ion concentration, and no doubt by other environmental influences (Wei et al., 1992). Weir et al. (1992b) concluded that nutritional status is the over-riding influence on *hrp* gene expression rather than plant derived compounds. Also associated with Fire Blight are genes termed *dsp*, which encode disease specific factors. Of these, DspE is secreted by an HRP secretory pathway (Bogdanove et al., 1998). In general, it is clear that the molecular biology of Fire Blight disease is beginning to be unraveled, however few details of the process are currently known.

Control of Fire Blight

The control of Fire Blight is a difficult and multifaceted task. Tools available to the orchardist include rigorous pruning regimes and the manual removal of over-wintering cankers, complex disease prediction models, and preventative chemical and biological treatments. Careful pruning can remove infected shoots if caught in time and if the severity of pruning is sufficient (Clarke et al., 1991). The susceptibility of shoots to infection can be reduced by Apogee® (prohexadione calcium), very recently released by the agrochemical arm of the company BASF®. Apogee inhibits the biosynthesis of gibberellin and reduces the growth of vegetative shoots which are susceptible to infection. To predict the onset of disease, Fire Blight risk assessment models have been developed using data from long term studies of conditions in which Fire Blight develops. The most important factors are warm temperatures and wet periods, which readily induce disease. Two Fire Blight risk assessment models can be found on the internet; Cougarblight 98C, www.ncw.wsu.edu/fbsmith.htm; and Maryblyt, www.intrepid.net/afirs/fb8.htm (19 January 2002). When the likelihood of disease is high, orchardists have a limited arsenal of preventative control methods (reviewed by Johnson and Stockwell, 1998; and Vanneste 1996). Chemical controls are applied by spraying and generally aim to target the bacterial population present on blossoms where most infections occur. These

controls include copper sulphate, which is limited by phytotoxicity against blossoms, and antibiotics such as streptomycin (marketed by Novartis as Agri-Mycin 17) and oxytetracycline (marketed as Terramycin). Streptomycin has been the Fire Blight control of choice due to its good efficacy and bactericidal mode of action, however strains of *E. amylovora* with resistance against streptomycin have arisen on numerous occasions (recently reviewed by Jones and Schnabel, 2000). Significantly, the use of streptomycin appears to reduce the natural microflora present in flowers resulting in more severe disease outbreaks in areas with streptomycin resistant *E. amylovora*. An alternative, oxytetracycline, is less efficacious than streptomycin as it is bacteriostatic and has a shorter half life in the field. Additionally, the use of antibiotics for control of fireblight has been banned in many European countries. In response to these limitations a substantial research effort has focused on an alternative control mechanism, which involves the suppression of *E. amylovora* by saprophytic epiphytes isolated from the blossoms of Fireblight susceptible plants. This approach, known as biological control, has had a checkered history in various ecological systems, and is the subject of much debate and speculation.

General aspects of biological control

Biological control is often thought of in the 'classical' sense as the introduction of a natural predator to control an exotic pest. In this sense, biological control has existed as a scientific discipline for over 100 years (Thomas and Willis, 1998). More recently, the National Academy of Sciences (US) defined biological control as 'the use of natural or modified organisms, genes, or gene products to reduce the effect of undesirable organisms (pests), and to favor desirable organisms such as crops, trees, animals and beneficial insects and microorganisms' (reported by Thomashow and Weller, 1996). A definition of a biological control agent (BCA) was proposed by the U.S Environmental Protection Agency (EPA) as 'any living organism applied to or introduced into the environment that is intended to function as a pesticide against another organism declared to be a pest by the administrator' (reported by Cook, 1996). These definitions show the broad scope of biological control in the modern setting and encompass the use of microbes as BCAs, the use of a native BCA against a native pest, and approaches such as isolating a BCA from the same niche as the pest and reapplying the BCA to diseased sites. Unfortunately, spectacularly unsuccessful applications of BCAs, particularly with regard to the unanticipated detrimental effects on non-target flora and fauna, have led to widespread skepticism towards biological control. However, because a successful biological control operation will never make as much noise as a significant failure, Cook (1996) suggested that it

is important that the real risks are assessed and that the perceived risks are not given an excessive influence on biological control decisions.

What does biological control offer that chemicals cannot? This comparison is particularly pertinent given that, in the US at least, a major hurdle in the development of BCA is licensing. Microbial BCA, but not other BCA such as insects or plants, are subject to the same rigorous and expensive registration process as chemicals. The use of chemicals to control pests is often (and sometimes realistically) perceived by the public at large as being unnatural and having undesirable side effects. Furthermore, Thomas and Willis (1998) suggest that chemical controls are usually short term, non-specific, and that alternatives are required to deal with the development of resistance (although the short half life of many agrichemicals can be desirable if non-target organisms are adversely affected). It has been suggested, somewhat controversially, that the use of microbial BCA for control of phytopathogens in agriculture and horticulture has many advantages over chemicals, including lack of resistance development and highly selective pathogen targeting (Dunne et al., 1996). Even if this is not true in every case, the manipulation of artificial agricultural 'ecosystems' by the informed use of microbial BCA can be seen as a more natural control process than the use of toxic chemicals.

Biological control is often hindered by incomplete pathogen suppression and on occasion by short term activity. Both usually result from mismanaged application, which itself results from a lack of research and development (Freckleton, 2000). Biological control failures usually result from inadequate colonization of the target site and variability in the activity of pathogen suppression mechanisms (Thomashow and Weller, 1996). The lack of funding for research and development into these aspects of biological control, along with a lack of implementation and the perception of users, are seen as major drawbacks with biological control (Cook, 1996). On this basis, Cook (1996) proposed that the increased use of microbial BCAs will lead to more familiarity and greater public acceptance of the methodology. Furthermore, Thomas (1998) suggested that criticism of biological control is not constructive without comparative data about chemical alternatives or doing nothing. Perhaps a more pressing concern in the development of many BCAs is the efficacy of pathogen suppression in the field, particularly in cases where the alternatives are limited or ineffectual, such as Fire Blight.

The suppression of a pathogen by another organism can result from one or more of three potential mechanisms; competition, predation, or the production of toxic chemicals (Powell, 1995). Both competition and the production of antibiotics by antagonistic microbes feature strongly in the suppression of the fireblight pathogen *E. amylovora*, as discussed below

(Johnson and Stockwell, 1998). In such cases, microbial biological control can ensure that toxic chemicals are produced in the same and often specialized niche as the pathogen, plus it offers the advantage of competitively excluding the pathogen from this niche. The synergistic effect of such an approach may require less chemical production for effective disease suppression than the use of chemicals alone. Furthermore, the use of a consortia of different antagonists with different suppressive activities in different conditions and the use of antagonists in combination with chemical controls are potential developments available to the biological control programme (Dunne et al., 1996).

Biological control of Fire Blight

The control of Fire Blight by the use of antagonistic microbes has been unequivocally demonstrated by a number of research groups both in New Zealand (Kearns, 1993; Vanneste et al., 1995; Vanneste et al., 1995), and elsewhere (Hattingh et al., 1986; Johnson et al., 1993; Rundle and Beer, 1987; Wilson et al., 1992; Wilson and Lindow, 1993; Wodzinski et al., 1994). Together with the fact that chemical treatments are of limited use, the control of Fire Blight by biological means is likely to play an important role in the future of apple and pear horticulture (reviewed by Johnson and Stockwell, 1998, 2000). Over the past ca. 35 years, research into the biological control of Fire Blight has progressed far. *Pseudomonas fluorescens* A506 has now been registered as a biological pesticide as Blightban A506® (Plant Health Technologies, Boise, ID), and *Erwinia herbicola* strain EhC9-1 is currently in the registration process. The history and development of the biological control of Fire Blight is an interesting model for biological control in general, and warrants a brief overview.

In 1967, Goodman demonstrated that the infection of an avirulent *E. amylovora* strain into the stem of apple shoots provided some degree of resistance against further infection, as did the injection of a saprophytic 'yellow' bacterium similar to *E. amylovora* (presumably a strain of *E. herbicola*). McIntyre et al (1974) subsequently showed that cell free sonicates of these organisms also acted analogously to a 'vaccine' presumably inducing a systemic protective response against all invading microbes. Unfortunately the response was generally not permanent, limiting the usefulness of such an approach in the orchard.

The use of a saprophytic antagonist of *E. amylovora* as a BCA was suggested by Erskine and Lopatecki (1975), after isolating a candidate organism, named *E. herbicola* 'Y' (yellow). They also suggested that *E. herbicola* strain Y prevents *E. amylovora* from accumulating a population sufficient to initiate and maintain a Fire Blight infection. This proposal was subsequently

supported by investigations that showed that *E. amylovora* multiplies exclusively on the stigma (Thomson, 1986), and that *E. herbicola* (Hattingh et al., 1986; Rundle and Beer, 1987; Wilson et al., 1992) and *P. fluorescens* A506 (Wilson and Lindow, 1993) multiply and occupy this same niche. For instance, Hattingh et al (1986) observed that *E. amylovora* and *E. herbicola* both grow on the stigma in the space between papillae, and proposed that both space and nutrients are a limited resource in this environment. In fact, competitive exclusion is a mechanism common to all Fire Blight biological control microbes isolated to date. For *P. fluorescens* A506 this is the only mechanism for *E. amylovora* suppression clearly demonstrated to date. The possibilities that A506 modifies the blossom environment to produce less nectar or modifies the plant compound arbutin to a toxic inhibitor of the pathogen (see below) have also been proposed (Wilson and Lindow, 1993).

The production of toxic chemicals is often associated with the suppression of *E. amylovora* by strains of *E. herbicola*. An early proposition was that β -glucosidase produced by saprophytic microbes such as *E. herbicola* caused the cleavage of arbutin to create hydroquinone, a compound shown to inhibit the growth of *E. amylovora* in plate assays (Chatterjee et al., 1969; Hildebrand and Schroth, 1964). Hildebrand et al. (1964) developed this proposal when they found that *E. herbicola* strains had predominantly strong β -glucosidase activity, whereas *E. amylovora* strains had only weak activity. Chatterjee et al. (1969) subsequently showed that hydroquinone inhibits oxygen uptake by *E. amylovora* and thus inhibits the growth of the Fire Blight pathogen.

More recent studies on the suppressive mechanisms of *E. herbicola* have demonstrated that the synthesis of toxic compounds, rather than the modification of plant compounds, is a common theme amongst strains of this species (El-Goorani and Beer, 1991; Hodges et al., 1980; Kearns and Hale, 1996; Wilson et al., 1990; Wodzinski et al., 1990; Wodzinski and Paulin, 1994; Wright et al., 2001). As summarized by Vanneste (1996), of ca. 1500 putative *E. herbicola* strains isolated in three studies in the 1990's, 38 % produced one or more antibiotics (El-Goorani and Beer, 1991; Wilson et al., 1990; Wodzinski and Paulin, 1994). The association of antibiotic production with disease suppression was shown in orchard trials involving twelve *E. herbicola* strains, from which four out of five antibiotic producing strains were excellent antagonists of Fire Blight, whereas seven antibiotic non-producing strains were weak antagonists (Wodzinski et al., 1994). Wilson et al. (1992) proposed that the exclusion of *E. amylovora* by *E. herbicola* strain HL9M3 involves competition and antibiosis because the growth of *E. amylovora* was inhibited by HL9M3 before nutrients became a limiting factor. More specifically, Vanneste et al (1996) demonstrated that a peptide antibiotic isolated from *E.*

herbicola strain Eh252 was inhibitory to *E. amylovora* using a fruit slice model for assessing Fire Blight suppression. This claim was strengthened by the finding that an antibiotic-minus mutant of Eh252 could not inhibit *E. amylovora* in the same assay system. They also proposed that in situ colonization was essential after finding that a derivative of *E. coli* DH5 α transformed to produce the antibiotic of Eh252 in vitro could not suppress *E. amylovora* using the fruit plug assay. Similarly, *E. herbicola* strain Eh318 produces two antibiotics thought to have a role in *E. amylovora* suppression since a spontaneous mutant of *E. amylovora* that was resistant to the two antibiotics was more weakly suppressed than the antibiotic-sensitive parent strain (Wodzinski et al., 1990; Wright et al., 2001). In summary, antibiotic production appears to have a role in the suppression of *E. amylovora* by *E. herbicola*; this is further discussed in Chapters 2 and 4.

A common and important problem associated with BCAs is good pest suppression in different environments. The Fire Blight biological control model has a potential solution to this problem. Since the activity of microbes varies with environment, various researchers have experimented with combinations of microbes with different antagonistic mechanisms, and with different optimal suppressive activity in different conditions. *P. fluorescens* A506 is best suited to cool temperatures whereas EhC9-1 is a better antagonist in warmer climates; therefore a mixture of both would be expected to offer control in both conditions (Johnson and Stockwell, 1998; Johnson et al., 1993). Similarly, A506 acts to colonize a stigma and exclude *E. amylovora* and as such needs to be applied two to three days prior to *E. amylovora* to offer control, whereas some *E. herbicola* strains produce one or more antibiotics that enable them to compete directly with *E. amylovora* for space and nutrients when co-inoculated (Wilson et al., 1992; Wilson and Lindow, 1993). The co-habitation of A506 and Eh252 was successfully demonstrated by Vanneste, thus a combination of A506 and Eh252 should enhance the ability of A506 to colonize the stigma of blossoms in the presence of *E. amylovora*. In attempting to test this prediction Vanneste et al (1995) found that the suppression of *E. amylovora* by Eh252 was not enhanced by the presence of A506, however the hypothesis was not truly tested as A506 by itself did not offer significant control in these experiments, which were carried out in an orchard in Hamilton, New Zealand. In contrast, most other trials involving A506 have been carried out in the US and demonstrated Fire Blight suppression sufficient to warrant the registration of this strain as a biological pesticide (Johnson et al., 1993; Lindow et al., 1996). This observation in itself highlights the validity of using a multi-strain consortia of *E. amylovora* antagonistic bacteria to ensure that suppression of the Fire Blight pathogen can be guaranteed in different environments.

The potential for inhibition of *E. amylovora* at the hypanthium was suggested recently by Pusey (2000). He proposed that the biological control capability of an antagonistic microbe may be influenced by the ability to tolerate nectar at the hypanthium surface. Since yeasts possessed higher osmotolerance than *E. amylovora*, which was higher than *E. herbicola*, Pusey (2000) proposed that saprophytic yeast strains may be worth developing as part of a consortia BCA.

Studies have also demonstrated that the integration of biological control agents with current control regimes may be a valid approach. As an example, Stockwell et al (1996) found that the establishment of A506 and EhC9-1 was not affected by co-application of streptomycin, although oxytetracycline treatment adversely affected the survival of these bacteria for several days. Lindow et al (1996) demonstrated that the application of A506 in combination with both streptomycin and oxytetracycline was additively better in reducing Fire Blight and frost damage than either treatment by itself.

A final feature required for successful integration of a biological control programme into orchard management schemes is the development of application methods that preferably fit in easily with current orchard practices. Again, the biological control of Fire Blight offers a good model for the successful development of such a system. The preparation of bacterial inocula was found to have a large influence of the successful establishment of the applied microbes (Stockwell et al., 1998). Stockwell et al (1998) found that lyophilized A506 established on significantly more flowers than bacteria suspended from agar plates. The reason for this was not clear, however the procedure reduces both variation in microbe establishment and provided an easily standardized method for preparation of a BCA that could potentially be stored long term. A second important aspect of orchard application is timing, for which complex disease forecast systems have been developed (Johnson and Stockwell, 1998; Eastgate, 2000). It has been shown that unopened blossoms rarely carry detectable populations of bacteria (>100 CFU.blossom⁻¹), and that populations develop in most flowers within days of opening (Johnson et al., 2000). According to Henis and Chet (1975), the introduction of potential antagonists is generally not successful because existing microflora prevent a new-comer from establishing. Thus, Johnston and Stockwell (1998) proposed multiple orchard applications from early blossom through to late blossom to ensure that populations of introduced saprophytes can colonize stigmas and exclude *E. amylovora* from becoming established.

Future biological control programmes to combat Fire Blight will most likely involve the use of a consortia of microbes, possibly with chemicals, to create a highly effective control agent with multiple suppressive activities (Johnson and Stockwell, 1998; Johnson and Stockwell, 2000).

All new strains are therefore useful, but will have to be screened for usage with current control methodologies. Furthermore, knowledge of the suppressive mechanisms and the modification of strains for enhanced biological control are important research areas required for the development of biological control strains to be incorporated in an integrated biological control programme.

The isolation and characterization of *E. herbicola* strain Eh1087

Erwinia herbicola strain Eh1087 was isolated by Kearns (1993) in 1989 during a survey of bacteria present on blossoms of apple trees (*Malus X. domestica* Borkh. cv. Golden Delicious) in Canterbury, New Zealand. It is worth noting that the species *E. herbicola* has been regrouped with some other *Erwinia* species as *Pantoea agglomerans*, however for continuity the name *E. herbicola* has been retained in the present study (Gavini et al., 1989). Eh1087 displayed superior ability to suppress strains of *E. amylovora* using *in vitro* and fruit slice assays than other bacteria isolated from the same trees (Kearns and Hale, 1995). Kearns subsequently used an immature pear fruit assay to show that antibiotic-minus mutants of Eh1087 did not suppress the ability of *E. amylovora* to produce Fire Blight symptoms (Kearns, 1993; Kearns and Mahanty, 1998). Once antibiosis was suspected of being involved in the suppression of *E. amylovora* by Eh1087, investigations into the genetic basis underlying antibiotic production by Eh1087 (Kearns and Hale, 1996), and the nature of the antibiotic were initiated (Kearns, 1993; Kearns and Mahanty, 1998). Further details of these investigations are presented in Chapters 3 and 4 respectively.

Objectives of the present investigation.

Control of Fire Blight is an important issue for the pip-fruit industry both in New Zealand and worldwide as the disease causes significant economic losses. The isolation of the *E. amylovora* antagonist *E. herbicola* Eh1087 from a Canterbury orchard is particularly relevant to the potential biocontrol of Fire Blight in the dry, cool growing climates associated with New Zealand East Coast orchards. Eh1087 is a good proposition as a biological control agent against *E. amylovora* as it appears to strongly inhibit the pathogen *in planta*, and it is adapted to New Zealand conditions. It is highly probable that the most effective biological control of Fire Blight will involve a consortia of various bacterial strains that possess different antagonistic properties (Johnson and Stockwell, 1998). Thus, the fact that Eh1087 has different properties to other potential Fire Blight control strains means that Eh1087 will be a useful strain to develop as part of such a consortia (Kearns, 1993; Kearns and Hale, 1996; Kearns and Mahanty, 1998). An understanding of the disease control mechanisms of Eh1087 at a fundamental level is essential

for such development. Once the significance of a control mechanism is established, modern molecular biology technologies offer the means to make substantial improvements to a biological control agent at the genetic level. Furthermore, the purified 'biocontrol metabolite' may itself have use in a Fire Blight control programme, and the characterization of such compounds may provide leads for the development of analogous active compounds.

1. Preliminary investigations by Kearns had indicated that the production of antibiotic was essential for suppression in a fruit slice assay (Kearns, 1993; Kearns and Mahanty, 1998), however there is evidence that this is a poor measure of efficacy *in planta* (Wilson et al., 1990). Therefore, a key objective of this investigation was to attempt to determine whether or not antibiotic production was an important mechanism for suppression of *E. amylovora* by Eh1087 *in planta*.

2. The main objective of the present study was to investigate the genetic determinants required for antibiotic production by Eh1087. A locus involved in this process had been located, however no information regarding the extent of the locus, or the genes and their expression was known (Kearns, 1993; Kearns and Mahanty, 1998).

3. Kearns and Hale (1996) demonstrated that crude extracts of the antibiotic produced by Eh1087 had different properties to antibiotics produced by other potential *E. herbicola* biological control agents, however the chemical structure of the antibiotic was not known. Thus, the final significant objective of this investigation was to purify the antibiotic and determine its structure, for comparison with other *E. herbicola* antibiotics, and to relate chemical structure to the potential functions of genes involved in its synthesis. An additional part of this investigation was to investigate the properties of the antibiotic with a view to determining its mode of action.

Chapter 2. THE ROLE OF AGA IN SUPPRESSION OF THE FIREBLIGHT PATHOGEN

Introduction

The biological control of Fire Blight will most likely involve a consortia of numerous complementary microbes (Johnson and Stockwell, 1998; Vanneste, 1996). The isolation of microbes suitable for use in such a consortia requires a simple and reproducible laboratory assay that is predictive of Fire Blight suppression in the field. Since *E. amylovora* grows on slices of immature pear and apple fruits and produces a sticky exudate considered representative of Fire Blight disease, an assay was developed to assess the ability of antagonistic bacteria to inhibit exudate production (Beer and Rundle, 1983). This assay was used to demonstrate the suppression of *E. amylovora* by *E. herbicola* strain EhC9-1, and by crude preparations of antibiotic from cultures of this strain (Ishimaru et al., 1988). The fruit slice was further developed as a quantitative assay based on the proportion of infected slices, and the severity of ooze production (Wilson et al., 1990). However, Wilson et al (1990) reported that the fruit slice assay gave inconsistencies in measurement of strain efficacy from one assay to the next, and that the assay does not provide a measure of Fire Blight suppression in blossoms. For example, of 34 *E. herbicola* isolates assessed using a pear slice assay, the two most suppressive strains and a very poor suppressor chosen as a control strain were subsequently found to have similar efficacy in planta. Inconsistencies between the pear slice assay and in planta assays were also noted for a selection of potential Fire Blight control strains from different genera (McLaughlin and Roberts, 1993). Vanneste (1996) argues that the fruit slice assay is valid as a qualitative assay, if carried out with care. He has further developed the fruit slice assay using small plugs of pears which reduced between-assay variation, since many plugs could be obtained from a single fruit (a potential source of variation)(Vanneste et al., 1996).

The biological control of *E. amylovora* occurs when the stigmatic surface of a blossom is colonized by an antagonist resulting in the exclusion of the pathogen (Johnson and Stockwell, 2000). There appears to be little information relating the environment of a fruit slice to the stigma of a flower where *E. amylovora* and antagonistic saprophytes interact (Hattingh et al., 1986). Because these environments are probably considerably different, and since the degree of antagonism in the fruit slice assay is not necessarily predictive of suppression in blossoms,

(Wilson et al., 1990), the efficacy of each potential Fire Blight control strain currently needs to be assessed in planta (Johnson and Stockwell, 2000). Wilson et al (1992) used an assay that involved competition between *E. amylovora* Ea519 and *E. herbicola* EhHL9N13 on the stigma of hawthorn flowers on branches maintained in controlled conditions. They found that the population of Ea519 was significantly reduced when the stigma was pre-inoculated or even co-inoculated with EhHL9N13. EhHL9N13 produces a broad-spectrum antibiotic that is active against *E. amylovora* on potato-dextrose agar plates, however since antibiotic-minus mutants were not competed against *E. amylovora* in the *in planta* assay, the influence of the antibiotic in Fire Blight suppression was not directly measured. Furthermore, Wilson et al (1992) did not assess the ability of EhHL9N13 to colonize flowers on which Ea519 had already established, which has relevance to the timing of orchard application. When resources are available, the efficacy of a biological control agent can be assessed in large scale orchard trials such as those carried out by Johnson et al (1993), who assessed the inhibition of *E. amylovora* spread by bees to flowers that had been pre-inoculated with *P. fluorescens* A506 or *E. herbicola* EhC9-1.

Large scale orchard assays are impractical for screening saprophytes for potential Fire Blight control strains. To simplify these assays, but maintain *in planta* realism, Pusey (1997) developed an alternative assay based on the competition between *E. amylovora* and an antagonist on the stigma of detached flowers. Pusey also manipulated the environment of pear trees to obtain blossoms almost year-round. Since the suppressive activity of twelve antagonists against *E. amylovora* on the detached flowers was not significantly different to that suppression in whole trees both in glass houses and in the field, this assay appears to be a good measure of the efficacy of a potential biological control strain (Pusey, 1997). Very recently, Mercier and Lindow (2001) developed an alternative method for rapidly assessing the populations of *E. amylovora* on numerous flowers. They created an *E. amylovora* derivative strain containing an ice nucleation gene (*iceC*) from *Pseudomonas syringae* that caused chilled flowers to freeze at higher temperatures than they would otherwise. The number of flowers frozen at a set temperature provided an approximate measure of the *E. amylovora* population present on a flower, and therefore the influence of an antagonist on that population (Mercier and Lindow, 2001).

The current investigation was designed to test the hypothesis that antibiotic production by Eh1087 is important for suppression of *E. amylovora* strain Ea8862. Strong support for this proposition was provided by Kearns (1993) who showed that, in contrast to Eh1087, antibiotic-minus mutants of Eh1087 do not prevent the development of fireblight symptoms on immature pear slices. The first question that remained to be answered was whether or not Eh1087

produces antibiotic (AGA) in the environment provided by sliced immature fruit. If not, then this would suggest that AGA production is not required for Ea8862 suppression but is linked to some other factor that is required for such suppression such as competition. A second question was whether or not AGA production was important for suppression of Ea8862 on the stigma of apple blossom, a situation more relevant to the environment in which these organisms compete in nature.

Methods

Extraction of AGA from apple slices

Apple slices were inoculated with Eh1087 as described by Kearns (1993), and sampled destructively every day for five days. At each sampling point, apple slices were suspended in five ml 1-A minimal salts, vortexed for 30 s, and sonicated for 10 s with a Transon Ultrasonic Cleaner at the top setting (6), before a 10 µl sample was taken to enumerate CFU. The remaining medium was centrifuged to remove solid debris, and extracted with 5 ml chloroform as described in chapter 4. The chloroform layer was removed and dried. The chloroform-extracted solids were dissolved in 50 µl 1-A medium and assessed for antibiotic activity against a lawn of Ea8862 grown on LB. The efficiency and reliability of the procedure was calibrated using apple slices spiked with a measured amount of stock AGA extract.

Blossom assays

Blossom assays were carried out in a similar manner as that described by Pusey (1997). Unopened blossoms were collected from *Malus x domestica* during September 2000, and individually placed in eppendorf tubes containing 10 % sucrose (Fig. 2.1A). Bacterial strains Ea8862, Eh1087, and EhΔAGA (Table A1), were grown overnight in LB with full antibiotic selection, and resuspended to the following concentrations (CFU.ml⁻¹) in phosphate buffer (10 mM, pH 7.0) containing 0.03 % Tween 20; Ea8862, 1 x 10⁷; Eh1087, 1 x 10⁸, EhΔAGA, 1 x 10⁸. One microlitre of suspended cell solution, containing 10⁴ or 10⁵ CFU, was placed on each stigma of a blossom using a Gilson pipette (Fig. 2.1A). The blossoms were arranged in a rack placed on moist towels in an air tight container, and incubated at 25 °C. After 0, 12, 24, 48, and 72 h, 24 flowers from each treatment were sacrificed to enumerate bacteria. The sample size was subsequently reduced to eight groups of three flowers. For each blossom the stigmas and styles were aseptically removed from the remainder of the flower parts (Fig. 2.1B). The stigma and styles from three blossoms were placed in an eppendorf tube containing one ml of 1-A medium with 10 % glycerol. Bacteria were separated from the stigma by 10-15 s vigorous vortexing and 60 s sonication, and immediately frozen at -80 °C. To enumerate the bacteria present on the stigmas, each frozen sample was thawed, diluted and plated on LB media supplemented with the appropriate antibiotic as follows; Ea8862, Nal; Eh1087, Rf; and EhΔAGA, Rf and Sm. Blossom assay data was analyzed using S-Plus version 4.5 (Mathsoft Inc.) with assistance and expertise provided by Gary Houliston, PAMS Dept., University of Canterbury, New Zealand.

Results

2.1. Eh1087 produces AGA when grown on apple slice

Eh1087 was chosen from a pool of orchard isolates because it demonstrated consistently strong suppression of *E. amylovora* in an immature pear slice assay (Kearns and Hale, 1995). Antibiotic-minus mutants of Eh1087 did not inhibit *E. amylovora* strain Ea8862 in this assay demonstrating the importance of AGA for the suppression of Ea8862 in an accepted, albeit artificial, fireblight assay system (Kearns and Mahanty, 1998). Two of the early goals of the present investigation were to confirm that Eh1087 produced AGA during the immature fruit assay, and to determine the timing of AGA production by Eh1087 in this environment. A simple procedure was developed for the recovery of AGA from an immature apple slice, which allowed the recovery of ca. 20 to 45 % of the AGA applied to an apple slice (see materials and methods). The extraction of AGA from apple slices inoculated with Eh1087 yielded AGA over the first 36 to 48 hours of the assay, as the Eh1087 population size increased (data not shown). Generally, greater than 10^8 Eh1087 cells per slice were required for AGA to be detected. After 48 hours the Eh1087 population stabilized and no AGA could be detected. No antibiotic activity could be extracted from apple slices in which the AGA-minus Eh1087 derivative Eh Δ AGA was grown, confirming that AGA produced by Eh1087 was isolated by the extraction procedure. The timing of AGA production by Eh1087 grown on immature apple slices is similar to that of broth cultures (see section 4.1.1) and supports the proposal that periods of population increase or growth are required for AGA production by Eh1087.

2.2. Competition between Eh1087 strains and Ea8862 on the stigma of apple blossoms

2.2.1. Experimental design

The stigma is the proposed site of interaction between *E. amylovora* and *E. herbicola* (Stockwell et al., 1998; Thomson, 1986; Wilson et al., 1992). To investigate the role of AGA in the suppression of Ea8862 by Eh1087 in a situation more representative of the real world than the fruit slice assay, an apple blossom assay similar to that described by Pusey (1998) was undertaken. Initially, a defined inoculum of two bacterial strains was applied to the stigma of apple blossoms that were individually maintained in 10 % sucrose in the laboratory. Recovery and enumeration of the bacteria from the blossoms at various time points gave a measure of the outcome of competition between the two strains. Eh1087 was expected to produce AGA in the conditions of the blossom assay since the populations of Eh1087 on apple blossoms were shown to multiply rapidly during petal drop (Kearns and Hale, 1995), and antibiotic production had coincided with Eh1087 growth *in vitro* (see Chapter 4). Additionally, petal drop was induced

early in cut flowers in which senescence is sped up, which could have enhanced the growth of Eh1087.

During the first season, a preliminary blossom assay was carried out that retrospectively served to demonstrate the large variation in bacterial populations between flowers. On the basis of the data generated in the first assay, a second assay was designed and carried out in which each sampling point was composed of eight replicates of three flowers (24 flowers total) for each treatment. Flowers were sampled as described in materials and methods (Fig. 2.1). The influence of AGA on the growth of Ea8862 was assessed by comparing populations of Ea8862 that had competed against Eh1087 with populations of Ea8862 that had competed against Eh Δ AGA. The importance of stigma colonization in the interactions between Ea8862 and Eh1087 or Eh Δ AGA was also assessed by varying the time at which flowers were inoculated with each strain. Three concurrent assays were carried out: A) Eh1087 or Eh Δ AGA were applied 24 hours before Ea8862; B) all bacteria were applied together; C) Ea8862 was applied 24 hours before Eh1087 or Eh Δ AGA. Binomial data generated from these experiments is presented in Appendix A5. The binomial data generated by this experiment were analyzed using a generalized linear model to compare the influence of AGA production (Eh1087) and competitive exclusion only (Eh Δ AGA) on the proportion of bacterial cells on the stigma that are Ea8862 (over time).

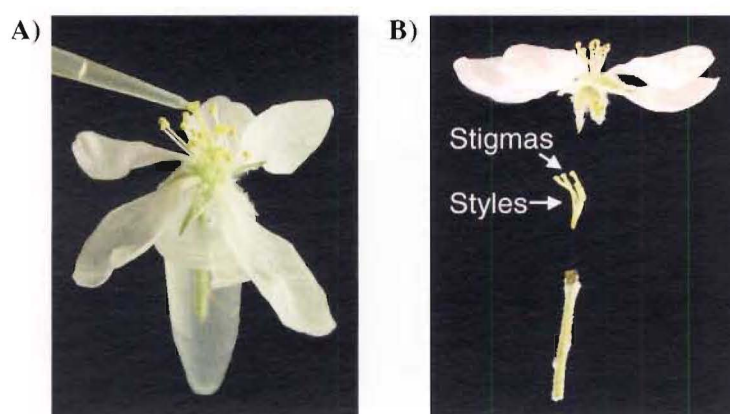


Figure 2.1. Stigma inoculation and sampling

A) One microlitre of a bacterial suspension was placed on the stigma of apple blossoms maintained in an eppendorf tube containing 10 % sucrose. B) Separation of the stigmas and styles from other flower parts.

2.2.2. AGA production does not disadvantage the growth of Eh1087 on stigma

Before the influence of AGA production on Ea8862 suppression was determined it was necessary to compare the population growth rates of Eh1087 and Eh Δ AGA to assess whether or not the production of AGA is disadvantageous *in planta*. The increase in populations of Eh1087 and Eh Δ AGA did not vary significantly over the 72 hour course of the experiment, indicating that AGA production did not influence the growth rate of Eh1087.

2.2.3. AGA production by Eh1087 has a significant role in the suppression of Ea8862

The ability of Ea8862 to colonize stigma already populated with Eh1087 was significantly lower ($P < 10^{-6}$, slope = -3.6) than the colonization of a stigma colonized with the AGA-minus mutant Eh Δ AGA. Furthermore, there was no significance difference in the effect of antibiotic production over time ($P = 0.3$). Interestingly, Ea8862 still struggled to get established on stigma populated with Eh Δ AGA, suggesting that once established Eh1087 may exclude competing strains by antibiosis *and* competitive exclusion (Fig. 2.2). When Eh1087 strains and Ea8862 were co-inoculated onto stigmas (Assay B), AGA production was again found to play a significant role in the suppression of Ea8862 ($P < 10^{-7}$, slope = -3.7). Assay B contrasted with Assay A in that there was a significant positive effect of AGA production over time ($P < 10^{-7}$), although this effect was weak (slope = +0.05). This finding indicates that AGA production is particularly influential when Ea8862 and Eh1087 are competing to establish in the same niche and is clearing illustrated in Figure 2.2 (Assay B).

2.2.4. Eh1087 cannot rapidly colonize a stigma populated with Ea8862

When stigmas were inoculated with Ea8862 24 h before the introduction of Eh1087 or Eh Δ AGA (Assay C), the population of Ea8862 increased only slightly once these strains were added (Fig. 2.2). In this situation, the influence of AGA production was not significant ($P = 0.4$), demonstrating that once Ea8862 is established on the stigma, the potential to produce AGA does not give Eh1087 any advantage over Eh Δ AGA in colonizing the stigma. Because two lines of evidence suggest that Eh1087 produces AGA during periods of growth (sections 2.1, 4.1.1), it is possible that the population of Eh1087 increases too slowly during colonization of an already populated stigma to produce sufficient AGA for significant reduction of the Ea8862 population. This is particularly likely given that the population of Eh1087 increases more slowly and to a smaller final size during Assay C than in either Assay A or B in which unpopulated stigma offered a niche for greater Eh1087 population increase (Fig. 2.2). Unfortunately, AGA production by Eh1087 during growth on the stigma was not assessed due to experimental difficulties.

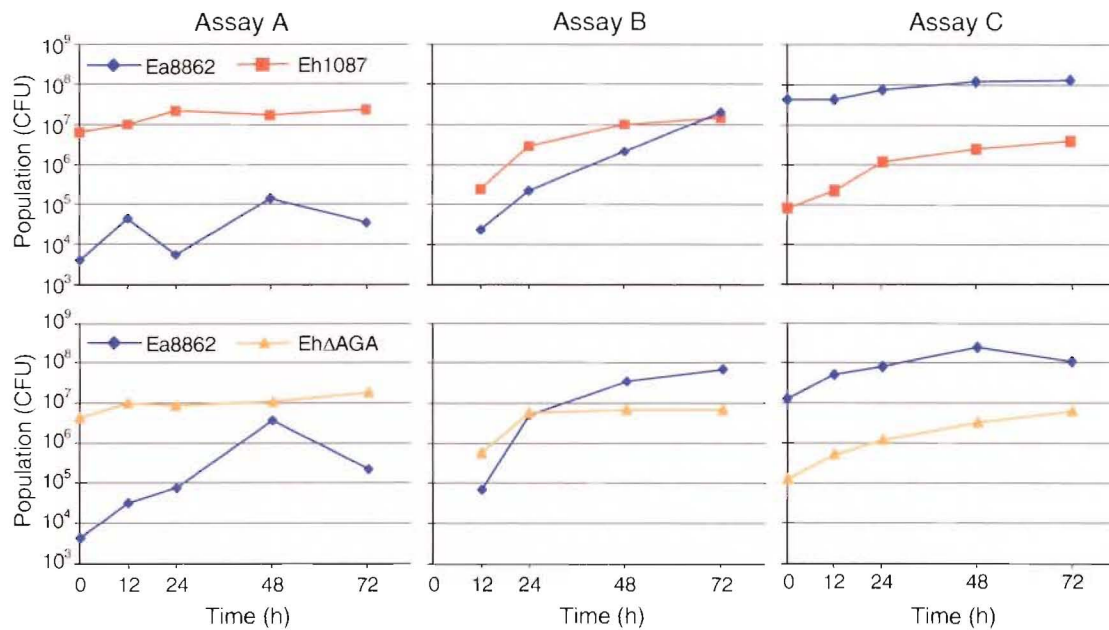


Figure 2.2. Competition between bacteria on the stigmas of apple blossom

The change in populations of Ea8862, Eh1087, and EhΔAGA grown in competition on the stigma of apple flowers. Data is reported in Appendix A5. Each data point represents the average of eight replicates of the bacterial population present on stigmas from three different blossoms.

Assay A: Eh1087 or EhΔAGA were inoculated 24 h before Ea8862.

Assay B: both competing bacteria were co-inoculated.

Assay C: Ea8862 was inoculated onto stigma 24 h before Eh1087 or EhΔAGA.

Assays A and C were sampled once the stigma were inoculated with the second bacterial species, whereas Assay B was assayed from 12 h after both bacterial species were placed on the stigma.

Discussion

Fruit slice assays may be inappropriate for the analysis of Eh1087 efficacy

The efficacy of Eh1087 as an antagonist of *E. amylovora* strain Ea8862 was previously measured using plate assays, and an immature fruit assay (Kearns, 1993; Kearns and Hale, 1995). Eh1087 was shown to produce an antibiotic that formed large clear zones of inhibition against lawns of Ea8862 grown on nutrient media plates (for example see Fig. 4.5), and Eh1087 antibiotic-minus mutants of Eh1087 did not suppress the development of fireblight symptoms in immature pear slices treated with Ea8862. However, the production of AGA by Eh1087 cultured on fruit slices was not assessed. During this investigation the development of methods to isolate and concentrate AGA led to the demonstration that this bacteria does produce AGA when grown on immature apple slices. However, antibiotic was only detected during periods of growth similar to the production of AGA by Eh1087 grown in broth cultures (see section 4.1.1). Ea8862 that were not killed during the growth period of Eh1087 can readily re-establish on the fruit and eventually cause disease symptoms, such as oozing tissue, to develop. For this reason data from an immature fruit assay needs to be interpreted carefully with relation to the timing of AGA production and *E. amylovora* suppression. For instance, on the basis of a similar immature fruit assay, Vanneste *et al.*, (1996) proposed that Eh1087 was significantly worse at suppressing *E. amylovora* than two other strains of *E. herbicola*, EhC9-1, and Eh252. However, given that the flesh of fruit is not the probable site of interaction between Ea8862 and Eh1087, and that the fruit tissue appears to poorly support the growth of Eh1087, this assay offers no real advantage over the plate assay to assess the suppression of *E. amylovora* by *E. herbicola*.

The role of AGA production in Ea8862 suppression assessed using a stigma-based assay

The role of AGA production by Eh1087 in the suppression of Ea8862 was assessed in a blossom assay more representative of the niche in which these bacteria compete in the orchard than the fruit slice assay (Pusey, 1997; Thomson, 1986; Wilson *et al.*, 1992). The stigma is a moist, nutrient rich environment that supports the growth of many bacteria, whereas few bacteria are found on other flower parts (Stockwell *et al.*, 1999). It is here that *E. amylovora* are thought to multiply, before moving down the style to the hypanthium to initiate a fireblight infection (Thomson, 1986). The multiplication of *E. amylovora* on the stigma can, however, be inhibited by competition from other epiphytic bacteria, particularly species of *E. herbicola* (Hattingh *et al.*, 1986; Rundle and Beer, 1987; Wilson *et al.*, 1992). Since the stigma is the probable site of interaction between *E. amylovora* and *E. herbicola*, it was a logical environment in which to assess the role of AGA in the suppression of *E. amylovora* Ea8862.

As with other bacteria investigated for their fireblight biocontrol potential, Eh1087 was found to multiply rapidly and fully populate a stigma with ca. 10^7 cells within 24 h (Fig. 2.2). AGA production was not assessed on blossoms. However, since Eh1087 multiplies readily on stigmas it is likely to produce AGA since AGA production correlated with periods of growth both in broth cultures (section 4.1.1.) and in immature fruit (section 2.1). Additional evidence for AGA production in planta is provided by the significant difference in suppression of Ea8862 by Eh1087 and Eh Δ AGA (see below), which are isogenic except for the presence of the AGA gene cluster in Eh1087.

To determine whether or not the production of AGA was necessary for suppression of Ea8862 in planta, populations of Ea8862 competed against the potential AGA-producer Eh1087 were compared with Ea8862 populations competed against the AGA-minus mutant Eh Δ AGA. In this regard the term suppression refers to the population of Ea8862 and not to the development of disease symptoms, however it is generally accepted that the risk of fireblight infection increases as the population of *E. amylovora* on stigma increases (Johnson and Stockwell, 1998). The role of AGA production was also compared with all other suppressive influences (including competitive exclusion), by enabling either the pathogen or the antagonist to populate the stigma for 24 h before introducing the competing bacteria. Two findings suggest that primary succession of a new stigma is a crucial process in determining the efficacy of biocontrol by Eh1087. Firstly, stigma populated with Eh Δ AGA were poorly colonized by Ea8862, and secondly, stigma populated with Ea8862 were poorly colonized by Eh1087. AGA production significantly improves the antagonistic properties of Eh1087 relative to Eh Δ AGA, but only when competing to populate new stigmatic surfaces, or for the exclusion of invading bacteria once a stigma is colonized. Similarly, the antibiotic-producing *E. herbicola* strain EhHL9N13 also suppressed *E. amylovora* during pre-emptive and competitive stigma colonization (Wilson et al., 1992). In contrast, the suppression of *E. amylovora* strain Ea8R was only significantly reduced by if stigma were pre-inoculated and not co-inoculated with *P. fluorescens* A506 (Wilson and Lindow, 1993). By implication, antibiotic production may give *E. herbicola* an advantage during competition for colonization of the stigma. AGA production does not enable Eh1087 to colonize Ea8862-populated stigma significantly better than Eh Δ AGA, but Ea8862 suppression can happen in the absence of AGA production (Fig. 2.2), competitive exclusion appears to be more a more important factor in Ea8862 suppression than AGA production.

A common proposal that has emerged from competition experiments involving *E. amylovora* and antagonistic epiphytes is the need to populate the stigma with the antagonist as soon after anthesis (flower opening) as possible (Johnson and Stockwell, 1998; Johnson et al., 2000;

Stockwell et al., 1999; Wilson et al., 1992). When blossoms first open there is little indigenous microflora present on the stigma. The microflora eventually become established when vectors, such as bees, come into contact with newly opened flowers. However, rather than relying on natural vectors to transfer bacteria applied to early opened flowers to late opened flowers, Johnson and Stockwell (1998) recommended application of antagonistic bacteria at least twice during flowering. It is expected that effective fireblight suppression by Eh1087 would also require application of Eh1087 to flowers as soon as possible after anthesis, since AGA production by Eh1087 gives a significant competitive advantage when pre- and co-inoculated with Ea8862, but does not enable Eh1087 to out-compete Ea8862 that are already established on a stigma. The ability of Eh1087 to suppress co-inoculated Ea8862 may increase the period in which the bacteria can be applied to orchards and offer effective protection against fireblight relative to the commercially available fireblight control strain, *Pseudomonas fluorescens* strain A506 (BlightBan A506, Plant Health Technologies, Boise, ID) which inhibits *E. amylovora* when pre-inoculated but not when coinoculated (Wilson and Lindow, 1993).

There remains the untested possibility that isolated and concentrated AGA, applied with Eh1087, may kill the indigenous microflora present on a flower, and enable Eh1087 to become established. This approach could potentially reduce orchard treatments to one application reasonably late in the bloom. Similarly, Johnson et al (2000) found that the co-application of streptomycin and *E. herbicola* strain C9-1S (Sm^R) increased the proportion of C9-1S relative to the indigenous flora, however this effect was lost in orchards that had been treated with streptomycin for many years. The increasing emergence of streptomycin resistance amongst bacterial species present in the orchard, both in *E. amylovora* (Lindow et al., 1996; Stockwell et al., 1996; and references therein) and in other indigenous microflora (Johnson et al., 2000), is a major issue in the control of fireblight. In this regard, Eh1087 and AGA, a new antibiotic that has not been used artificially to control the Fire Blight pathogen, could offer a useful alternative to the currently used control measures. The combined application of Eh1087 with purified AGA to remove bacterial competitors and aid the establishment of Eh1087 during flowering is a logical approach but one that requires further investigation.

The current investigation confirmed the significance of antibiosis as a mechanism for the suppression of *E. amylovora* by Eh1087 in situ. Thus, further investigation of the antibiotic and the genes required for its synthesis were warranted. The development of Eh1087 as a BCA however, requires further investigation. The efficacy of EH1087 has not been demonstrated in orchard trials, and the strain has not been assessed for ice-nucleation activity or induction of fruit russetting, features that may exclude Eh1087 as a useful BCA (Johnson and Stockwell,

1998). Assuming Eh1087 has favorable properties and good efficacy in the orchard, its use as a BCA would also require the assessment of methods for orchard application involving current orchard practices. Furthermore, the use of Eh1087 with other Fire Blight antagonists, and the isolation of strains able to resist current chemical Fire Blight controls needs to be assessed (Vanneste et al., 1995; Vanneste et al., 1995). The findings presented in the following Chapters indicate that Eh1087 is suitable for development by molecular methods for enhanced and extended antibiotic production in various conditions.

Chapter 3. A GENETIC ANALYSIS OF AGA PRODUCTION BY EH1087

Introduction

Modification of a biological control agent by molecular methodologies may be of particular use in the development of a Fire Blight control regime, in which it is proposed that a consortia of different agents with different suppressive mechanisms will be the most successful approach (Johnson and Stockwell, 1998; Johnson and Stockwell, 2000; Vanneste, 1996). As stated by Dunne et al (1996) 'the cloning and characterization of genes involved in the production of a biocontrol metabolite offers possibilities for the improvement of biocontrol agents'. However, before a biological control strain can be improved by genetic manipulation, the genetic basis underlying the suppression mechanism needs to be fully characterized. Pathways that have been sufficiently described may have the potential to be manipulated to increase antibiotic production, or the range of environments in which the organism produces antibiotic, or even to create novel antibiotics.

The antibiotic produced by Eh1087 has different properties to all other currently described antibiotics produced by *E. herbicola* strains (discussed in Chapter 4). Kearns (1998) had also found that four putative linked genes were involved in antibiotic production by Eh1087 suggesting that a cluster of genes are involved in this process. Therefore, as this chapter presents an investigation into the genes required for antibiotic production by Eh1087, it is useful to look at antibiotic production by micro-organisms in general to find common features of antibiotic gene clusters. Almost all known naturally synthesized antibiotics are produced by prokaryotic actinomycetes or eukaryotic fungi, and the antibiotic biosynthesis pathways in these organisms have received considerable attention due to the pharmaceutical value of their products (Kirby, 1992). General themes in the organization of genes for antibiotic synthesis have emerged. Such genes are usually organized in clusters, and in some cases as subclusters; both arrangements offer a mechanism to temporally coordinate the production of appropriate enzymes or enzyme complexes as they are required (Martin and Liras, 1989). Others have suggested that the structure of gene clusters arises from the evolutionary processes that created them (Lawrence and Roth, 1996). It has also been proposed that the ordered expression of the genes within a cluster enables each enzyme to act on the synthesized product of each earlier

enzyme, thereby reducing the randomness by which an enzyme locates its substrate (Martin and Liras, 1989). One or more resistance genes are often found within antibiotic biosynthesis gene clusters and are coordinately expressed with the biosynthesis genes to prevent the suicide of the organism producing the antibiotic. Finally, antibiotic production is usually affected by environmental conditions; for instance many antibiotics and other secondary metabolites are produced as resources for growth become exhausted and unwanted metabolites accumulate. Thus, the expression of antibiotic synthesis genes is often regulated by environmental signals, particularly during entry into stationary phase when there are insufficient resources for continued population increase.

Many antibiotic gene clusters have been isolated from species of *Streptomyces* (for examples see Bormann et al., 1996; Hong et al., 1997; Kakavas et al., 1997; McHenney et al., 1998). The first steps in characterizing an antibiotic gene cluster are to locate and clone it, and to attempt expression of the entire gene cluster in a heterologous host. A common approach is to screen a library of large, 20-50 kbp clones representing all the genomic DNA from an antibiotic producing organism, for the ability to produce antibiotic in a heterologous host. Because a gene cluster may not be correctly expressed in a new host organism, alternative approaches may be needed. McHenney et al (1998) located the Daptomycin gene cluster by determining the position of transposon insertions responsible for an antibiotic-minus phenotype in *Streptomyces roseosporus*. Bormann et al (1996) used a reverse genetics approach to clone a section of DNA required for the expression of two proteins involved in Nikkomycin synthesis. The similarities between at least some components of antibiotic pathways in species of *Streptomyces* enabled Kakavas et al (1997) to successfully isolate a polyketide synthase (PKS) from a Niddamycin gene cluster using a pair of degenerate primers based on PKS genes in other antibiotic gene clusters. Even more simply, Hong et al (1997) hybridized a PKS gene from *Streptomyces coelicolor* A3(2) to genomic libraries of various *Streptomyces* species to isolate clones containing cluster of PKS genes responsible for the synthesis of Tetrangulol and Tetrangomycin. Once gene cluster clones are isolated from a genomic library, they can often be expressed in a genetically tractable heterologous host such as *E. coli*, making further characterization and manipulation of these clusters far easier than it would otherwise be in *Streptomyces* species.

Of the antibiotic-producing *Erwinia herbicola* species with potential as Fire Blight antagonists, there is very little information about the genes involved in antibiotic synthesis. Examples of preliminary investigations into the genes involved in antibiotic production by *E. herbicola* are provided by Vanneste and Yu (1996) for strain Eh252, and by Wright et al (2001) for strain

Eh318. The DNA region required for antibiotic production by Eh252 was cloned and localized to a 2.4 kbp fragment (Vanneste and Yu, 1996). Therefore, it is unlikely that there are more than one or two genes involved in the production of this antibiotic, and since it is susceptible to protease, it may be a peptide antibiotic encoded by a single gene (Vanneste et al., 1992). Eh318 was originally thought to produce a single antibiotic (Wodzinski et al., 1990), however two different cosmids recently isolated from a genomic library of this strain each transformed *E. coli* DH5 α to produce antibiotics with different properties (Wright et al., 2001). These antibiotics were given the trivial names, Pantocin A and Pantocin B. Wright et al (2001) created a double mutant of Eh318 that could not produce Pantocin A or B. This mutant was not inhibitory to *E. amylovora*, but was inhibitory to a selection of different bacteria, demonstrating that Eh318 produces at least three antibiotics. However, for both Eh252 and Eh318, no genes or gene clusters required for antibiotic production have been characterized in any detail as far as the author is aware. An *Erwinia* species for which an antibiotic gene cluster has been described is *E. carotovora* strain GS101. GS101 contains a cluster of eight *car* genes that are responsible for the synthesis of a β -lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid (McGowan et al., 1997). Two of these genes, *carF* and *carG*, provided some resistance to the antibiotic (McGowan et al., 1996). As with other antibiotic gene clusters, each of these genes are transcribed in the same direction. This gene cluster was of particular interest at the beginning of the present investigation because Kearns and Hale (1996) had previously observed that the antibiotic of Eh1087 was inactivated by relatively high concentrations of β -lactamase, and predicted that it may have been a β -lactam antibiotic. However, since it is now known that Eh1087 produces a phenazine antibiotic (Chapter 4), a brief overview of phenazine gene clusters is warranted.

Investigations into the genetic and biochemical mechanisms responsible for phenazine production have so far focused on *Pseudomonas* spp., in which phenazine production is often important for the specificity and efficacy of these organisms as biocontrol agents. For instance, the production of phenazine-1-carboxylic acid (PCA) by *P. fluorescens* 2-79 is required for suppression of a variety of fungal diseases including take-all, a wheat-root disease caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller, 1988; Thomashow et al., 1990). Similarly, phenazine-1-carboxamide (PCN) produced by *P. chlororaphis* PCL1391 suppresses tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, however PCA produced by *P. fluorescens* 2-79 does not inhibit this disease (Chin-A-Woeng et al., 1998). A cluster of seven genes (*phzA* - *phzG*) cloned from *P. fluorescens* 2-79 by Mavrodi et al. (1998) was shown to be sufficient for PCA production. The transformation of *P. fluorescens* SBW25 with this gene cluster resulted in a strain that could produce PCA and was more

effective at reducing damping-off disease of pea seedlings caused by *Pythium ultimum* (Timms-Wilson et al., 2000). In a recent screening of phenazine-producing *Pseudomonads*, the seven-gene PCA operon was found in strains of *P. aeruginosa*, *P. aureofaciens*, *P. chlororaphis*, and *P. fluorescens* but not in isolates of *Burkholderia cepacia*, *Burkholderia phenazinium*, or *Brevibacterium iodinum*, suggesting that a second genetic mechanism may exist for the synthesis of the phenazine nucleus in these phenazine producers (Mavrodi et al., 2001). *P. aeruginosa* possesses two functional and nearly identical copies of the PCA gene cluster in different areas of the chromosome.

Mavrodi et al. (1998) proposed a biochemical model for the synthesis of the phenazine nucleus by the products of the PCA gene cluster that was supported, with some modifications, by the recent findings of McDonald et al. (2001). Initially PhzE catalyses the transfer of an amide nitrogen from glutamine to chorismic acid, creating 2-amino-2-deoxyisochorismic acid, which is converted to trans-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) by the protein PhzD. PhzF and PhzG are absolutely required for dimerization of two molecules of DHHA to create a phenazine nucleus in the form of PCA. The mechanism of this dimerization is currently unknown, although the data of McDonald et al. (2001) indicates that the dimerization reaction is enhanced by both PhzA and PhzB. An alternative mechanism was considered likely for synthesis of the phenazine nucleus in *Streptomyces* spp., although at this stage the genetic basis for this conversion has not been investigated (Van't-Land et al., 1993).

The PCA operon is present in many *Pseudomonas* species, however these species can produce many different types of phenazine antibiotics. These phenazines arise from modification of the common intermediate, PCA, by the product of one or more genes located outside the cluster of genes that make up the PCA operon. For instance, the *P. chlororaphis* *phzH* gene is required for the conversion of PCA to phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al., 2001). Similarly, *phzO*, encoded immediately downstream of the PCA operon in *P. aureofaciens* 30-84, and *phzS*, encoded downstream of PCA operon 1 in *P. aeruginosa* PAO1, are responsible for the hydroxylation of PCA to create 2-hydroxyphenazine-carboxylic acid (2-OH-PCA) and 1-hydroxyphenazine (1-OH-PHZ), respectively (Delaney et al., 2001; Mavrodi et al., 2001). In addition, the protein PhzS is required for the conversion of PCA to pyocyanin by *P. aeruginosa* PAO1 in association with the product of *phzM*, located immediately upstream of PCA operon 1. *P. aeruginosa* PAO1 also possesses a homolog of the *P. chlororaphis* *phzH* gene some distance from either of the PCA operons. Mavrodi et al. (2001) showed that *P. aeruginosa* PAO1 genes *phzH*, *phzS*, or both *phzS* and *phzM*, conferred on *E. coli* JM109 the ability to convert exogenous PCA into PCN, 1-OH-PCA, or pyocyanin, respectively. The potential for altering

phenazine production and therefore the range of organisms able to be suppressed by *Pseudomonas* spp. was demonstrated by Chin-A-Wong et al (2001) who transferred *phzH* from *P. chlororaphis* PCL1391 to *P. fluorescens* 2-79 creating a strain that could convert PCA to PCN and thereafter suppress tomato root rot.

During the present investigation Eh1087 was found to produce the phenazine antibiotic, D-alanylgriseoliteic acid (AGA, see Chapter 4). AGA was found to be active against a broad range of Gram-negative and Gram-positive organisms, including the Fire Blight pathogen *E. amylovora*. The finding that antibiotic production by Eh1087 was required for suppression of *E. amylovora* in fruit slice assays led to investigations into the genetic basis for antibiotic production by Eh1087 (Kearns, 1993). Initially, Kearns (1993) isolated six mutants defective for antibiotic production from 800 *TnphoA* insertion mutants. DNA adjacent to the *TnphoA* insertion in one of these mutants, EhA17g, was cloned and hybridized to a library of Eh1087 genomic cosmid clones. Of 13 cosmids that hybridized to this probe, six restored antibiotic production to EhA17g, however some variability in antibiotic production among the transformants was observed. Further variation in antibiotic production was observed in the remaining five mutants, EhA11g, EhA12e, EhA19f, EhA20f, and EhA46a, harboring cosmids pLA272, pLA215, or pLA255 (Fig. 3.1). Hybridization analyses also showed that the mutated locus was located on a ca. 200 kbp plasmid in Eh1087 (Kearns and Mahanty, 1998). Analysis of the DNA sequence in and around the locus revealed four potential open reading frames that had been interrupted by transposon insertions in various mutants. During the present study these ORFs and other genes associated with antibiotic production by Eh1087 have been designated *ehp*, for *Erwinia herbicola* phenazine, to differentiate them from the phenazine genes (*phz*) described in *Pseudomonas* species.

Initial research into the genetic basis for antibiotic production by Eh1087 by Kearns provided a good starting point for elucidating and describing the genetic region involved in antibiotic production. At the start of the present investigation, very little was known about the following: i) the extent of the region involved in antibiotic production, ii) the function, and regulation of expression of genes within this region, iii) whether this region is sufficient for antibiotic production or if genes outside this region are required for antibiotic production, or even iv) how Eh1087 protects itself from the antibiotic that it produces. These questions formed the basis for the investigations reported in this chapter.

Methods

Creation of Eh1087 mutants with defined transposon insertions

DNA from the region to be mutated was ligated into a suitable vector, usually pBR322, and subjected to random insertional mutagenesis with transposons mini-Tn10-LK, mini-Tn5*phoA*, or miniMudII [Kleckner et al., 1991; Castilho et al., 1984; de Lorenzo et al., 1990]. The resulting transposon insertions were mapped by analysis of restriction enzyme fragments, and plasmids containing appropriate insertions were introduced to Eh1087. Recombinant derivatives of Eh1087 were screened for loss of the plasmid-encoded antibiotic resistance marker and for maintenance of the transposon-encoded antibiotic resistance marker following homologous recombination between the introduced plasmid and the Eh1087 genome. When recombination was not successful using these plasmids, insertions were transferred, again by homologous recombination, to an appropriate cosmid in an *E. coli* *recA*⁺ host. Recombination between the cosmid carrying the transposon insertion and the genome of Eh1087 occurred at a high frequency because the large region of DNA either side of the transposon insertions enabled recombination to initiate readily. For all strains created by these processes, the predicted alterations in the restriction enzyme patterns due to transposon insertion were verified by an appropriate Southern hybridization (Appendix A1.5), and in some cases by restoration of the original Eh1087 genome by homologous recombination between the mutant genome and a plasmid containing the relevant region of wild-type Eh1087 DNA.

Creation and isolation of random Eh1087 AGA-minus mutants

Mini-Tn5*lacZ*2 transposon mutants of Eh1087 were generated using a simplified version of a published method (de Lorenzo et al., 1990). Briefly, miniTn5*km**lacZ*2 was introduced to Eh1087 on a suicide plasmid (pUT) by conjugation with *E. coli* S17-1 which possesses λ pir to enable plasmid replication and RP4 *mob* function for conjugative transfer of the plasmid to Eh1087 Rf^R recipient cells. Liquid cultures of *E. coli* S17-1 (pUT::mini-Tn5*lacZ*2) and Eh1087 were grown overnight, the cells rinsed twice in fresh liquid media, mixed, and plated on LB-agar plates supplemented with Rf, Km, and Xg. Because Rf has a bacteriostatic mode of action the *E. coli* donor cells were not killed and were able to donate pUT::mini-Tn5*lacZ*2 to the recipient Eh1087 (Rf^R) on the selective media. No more than 50 Eh1087 mutants from each conjugation were screened for loss of AGA production to ensure independent transposon-insertion mutants were isolated. If more than one mutant was isolated from a single conjugation, only those with obvious differences in colony morphology, AGA-production, or β -galactosidase activity were retained. Eh1087 transconjugants were transferred to Ea8862 lawns

grown on agar composed of rich media (LB) or defined minimal media (1-A), to isolate mutants unable to produce antibiotic when cultured on one or both one media types and to enumerate auxotrophic mutants. Antibiotic-minus mutants were subsequently purified on selective LB media. Each purified mutant was then cultured overnight in broth culture with full antibiotic selection and reassessed for antibiotic production. The remainder of each culture was divided; half was used to prepare genomic DNA, and half was stored at -80°C in LB with 10 % glycerol.

Insertion point cloning

To clone the section of DNA containing mini-Tn5*lacZ2* and the adjacent DNA from an Eh1087 mutant, genomic DNA prepared from each mutant was digested with *Pst*I or *Sal*I, which do not cut within the transposon, ligated to pBluescriptKS, and used to transform *E. coli* DH5 α to Ap^R Km^R. On occasion, the cloned DNA was further subcloned to provide suitable clones for DNA sequence determination.

Transposon insertion mapping

The position and orientation of transposon insertions within the *ehp* gene cluster was mapped by hybridizing cloned *ehp* gene cluster fragments to digested genomic DNA prepared from Eh1087 *ehp* mutants. Genomic DNA prepared from each Eh1087 mutant was digested to completion with restriction endonucleases *Eco*RI, *Bam*HI and *Eco*RI + *Bam*HI, separated by agarose gel electrophoresis and transferred to a Hybond N+ membrane. To screen for mutants with single transposon insertions the fixed DNA was hybridized to a 3.2-kbp *Not*I DNA fragment containing the kanamycin resistance gene present in mini-Tn5*lacZ2*. To localize the position and orientation of insertions in the Eh1087 *ehp* gene cluster to within 50-100 bp, cloned DNA fragments from pBB7, pBE5a, and pBE5b (Table 1) were hybridized to the fixed genomic DNA to detect differences in fragment sizes between Eh1087 and the *ehp* mutant derivative strains.

Minicell analysis

The sizes of the Ehp proteins were assessed by *E. coli* minicell analysis. Homologous recombination was used to transfer O1 transposon insertions from mutants *EhehpA*-*EhehpE* to pBB7, from *EhehpG*-*EhehpK* to pBE5a, and from *EhehpM*-*EhehpO* to pBE5b. The derived recombinant plasmids were introduced to the *E. coli* minicell strain P678-54T. Each strain was cultured in 400 ml LB for 5 - 6 h to an optical density of 1.0 at A₆₀₀. Vegetative cells were sedimented by centrifugation (1000 g, 10 min, 4 $^{\circ}\text{C}$) and discarded, and the remaining cells

were collected by further centrifugation (10000 g, 10 min, 4 °C). The cell pellet was resuspended in 2 ml BSG buffer (150 mM NaCl, 20 mM KH₂PO₄, 40 mM Na₂HPO₄, 0.1 g.l⁻¹ gelatin, adjusted to pH 7.4 with 2M KOH), and carefully laid on a linear sucrose gradient prepared by freeze-thawing 35 ml sterile BSG containing 20 % sucrose (Gibco BRL *ultraPure*) in clear 50 ml plastic centrifuge tubes. Sucrose gradients were centrifuged in an IEC model B-22M centrifuge using swing out rotor no. 960 to obtain a linear gradient (5000 g, 15 min, 4 °C). A 10 ml sample of the top band was carefully removed using a Jencons Powerpette Plus autopipettor and diluted with 10 ml BSG, and the cells were collected by centrifugation (10000 g, 10 min, 4 °C). The cells were resuspended in 2 ml BSG, and the cell separation process was repeated, before cells were finally resuspended to an optical density of 2.0 at A₆₀₀ in sterile minimal medium containing 30 % glycerol and stored at -80 °C as 100 µl aliquots. To radiolabel the proteins synthesized by the minicell strains, each 100 µl aliquot was added to 900 µl of M63 labeling buffer (M63 salts (Miller, 1972) containing all amino acids with the exception of cysteine and methionine) and incubated at 30 °C for 30 min, before two µCi Pro-Mix L-[³⁵S] (Amersham Pharmacia Biotech) was added and the incubation continued for a further 30 min. The labeled cells were collected by centrifugation (10000 g, 10 min, 4 °C) and resuspended in 60 µl buffer (70 mM NaCl, 20 mM KH₂PO₄, 50 mM Na₂HPO₄, and 1 mM MgSO₄) for storage at -80 °C. Radiolabelled proteins were separated by electrophoresis through either 12.5 % linear or 8 – 16 % gradient SDS-polyacrylamide gels, and protein sizes were estimated by comparison with a BioRad broad range protein standard.

Creation of EhΔAGA

To create EhΔAGA a 7 kbp *Bam*HI/*Eco*RI fragment of pLA272 DNA from upstream of the AGA gene cluster and a 5 kbp *Eco*RI/*Hind*III fragment of pLA267 DNA from downstream of the AGA gene cluster were ligated to a 1.8 kbp *Eco*RI fragment of DNA from pHRP315 containing a Ω cassette (Sm^R) (Parales and Harwood, 1993). This construct was ligated to *Bam*HI/*Hind*III digested pLAFR3 to create pLAΔAGA. pLAΔAGA was introduced to *EhehpA* (which contains an active *lacZ* fusion) and recombinants were screened for loss of β-galactosidase activity, loss of the vector pLAFR3 (Tc^R), and maintenance of Sm^R. Southern hybridization analysis of two resulting recombinant EhΔAGA strains demonstrated that the AGA-resistance and biosynthesis regions were missing whilst the flanking fragments of DNA and the Sm^R cassette were present in the predicted order (data not shown).

Colour production and complementation

To qualitatively assess the production of coloured AGA precursors, each *ehp* mutant was cultured in a microtitre dish well containing 100 µl of 2 % King's A medium as previously described (Carson and Jensen, 1974). Precursor feeding was carried out by placing 20 µg of AP1 isolated from EhΔAGA(pBB7) or AP3 isolated from E*ehpO* onto a 6 mm ϕ antibiotic disk, impregnating the disk with ca. 10^7 cells of an Eh1087 *ehp* mutant, and assessing antibiotic production on an Ea8862 lawn. Mutant-mutant complementation was carried out either by mixing overnight cultures of each *ehp* mutant combination and placing one microlitre on a lawn of Ea8862, or by placing one microlitre of each mutant 1-2 mm apart on a lawn of Ea8862. To assess DNA complementation, pLA272 or O2 mini-Tn5*lacZ2* insertion derivatives of this plasmid were introduced to representative E*ehp recA* strains and antibiotic production was measured.

Isolation and characterization of the Eh1087 *recA* gene.

A cosmid encoding the Eh1087 *recA* gene was isolated from an Eh1087 genomic library by transforming *E. coli* DH5 α with cosmid clones and selecting transformant with restored RecA-dependent DNA-repair mechanisms. Conditions sufficient for differentiation between *recA*⁺ and *recA*⁻ cells, were exposure to 0.125 mM MMS (methanesulfonic acid methyl ester), or 20 seconds UV irradiation in a Gelman Sciences Australia Biohazard using a Philips TUV 30W/630T8 lamp at 50 cm. To localize the *recA* gene for subsequent manipulations, *recA*-cosmid DNA (pL*ArecA*) was digested with *Pst*I, ligated to pBluescriptKS-, and used to transform DH5 α . Transformants were again selected for the ability to complement DH5 α *recA*. A 1.8 kbp *Pst*I fragment had the complete *recA* gene (plasmid pKS*recA*). DNA sequence was determined from each end of the cloned DNA in plasmid pKS*recA* using T3 and T7 primers.

Creation of Eh1087 *recA* strains

To mutate the Eh1087 *recA* gene, a chloramphenicol resistance marker was transferred into the *Eco*RI site between bases 777 and 778 of the predicted Eh1087 *recA* gene to create pKS*recA*777. The marked, interrupted Eh1087 *recA* gene was confirmed by its inability to complement DH5 α *recA*. Because of the high copy number of pKS*recA*777 and the short sections of DNA either side of the Ω Cm cassette, the mutated *recA* gene was transferred to an Eh1087 *recA* cosmid by homologous recombination in *E. coli* MC4100 *recA*⁺. Plasmid DNA prepared from this culture was introduced to DH5 α and transformants were selected for Tc^R and Cm^R to isolate a *recA*⁻ recombinant cosmid (pL*ArecA*777). To interrupt the *recA* gene of

Eh1087 strains, a process was developed for transfer of the $\Omega\text{Cm}^{\text{R}}$ marker from pL*ArecA777* to the relevant Eh1087 genome. Because the cosmid vector pLARF3 has an oriT site, pL*ArecA777* was introduced to *E. coli* S17-1 by electroporation, for subsequent RP4-mediated conjugal transfer to Eh1087 strains. Eh1087 *recA777* mutant-recombinants were initially screened for Tc^{S} indicating loss of the pLARF3 vector, and then for increased MMS and UV sensitivity relative to the parental strain to ensure that *recA* was inactivated. To check that the $\Omega\text{Cm}^{\text{R}}$ cassette was present in the *recA* gene of Eh1087 recombinants, genomic DNA from these strains was digested with *Pst*I, hybridized to the 1785 bp *Pst*I *recA* fragment of pKS*recA*, and assessed for an increase in size of 3.2 kbp due to the presence of the $\Omega\text{Cm}^{\text{R}}$ cassette.

Results

3.1. Antibiotic production and immunity are conferred by Eh1087 cosmids

One of the underlying goals of this investigation was the characterization of the genes and their role in the production of antibiotic by Eh1087. Four candidate antibiotic-production genes had already been implicated in antibiotic production in a previous investigation by Kearns (1993) who found that mutations within a 2.2 kbp region (region 1, Fig. 3.1) on a ca. 200 kb plasmid present in Eh1087 abolished antibiotic production. However, little was known about these genes or the extent of this region (Kearns and Mahanty, 1998). A resource for determining the extent of this region was a collection of 13 cosmid clones containing genomic DNA from Eh1087, created and isolated by Kearns (1993). During the present investigation, the mapping of restriction endonuclease fragments of cosmid DNA demonstrated that the 13 cosmids isolated by Kearns comprised nine different overlapping cosmids (Fig. 3.1). To determine whether or not the cosmids encoded sufficient information for AGA-production (hereafter referred to as the AGA-gene cluster) all nine cosmids were introduced to *E. coli* MC4100. MC4100 containing the cosmids pLA272, pLA305, pLA214, pLA215, or pLA424 produced antibiotic, however antibiotic production was detected when these strains were grown on minimal media but not on rich media (e.g. LB), varied from strain to strain, and was always less than Eh1087. In contrast, *E. herbicola* field isolate Eh262 harboring cosmid pLA214 produces a similar amount of antibiotic as Eh1087, both in rich and minimal media, suggesting that there may be *Erwinia*-specific factors or expression mechanisms that overcome the rich media suppression observed in *E. coli* MC4100. The finding that cosmids pLA255, pLA367, pLA179, and pLA101 do not confer antibiotic production on MC4100, demonstrated that the AGA-gene cluster must extend beyond the Eh1087 DNA region covered by these cosmids (Fig. 3.1). Additionally, cosmids pLA272, pLA305, and pLA214 provide MC4100 with resistance to AGA. These data demonstrated that information upstream of cosmid pLA255 was required for antibiotic production, and information upstream of cosmids pLA215 and pLA424 was required for AGA resistance (Fig. 3.1).

3.2. Boundaries of the region required for antibiotic production in Eh1087

The analysis of cosmids with sufficient information for antibiotic production and resistance demonstrated that the AGA-gene cluster was more extensive than the 2.2 kbp region (region 1, Fig. 3.1) reported previously (Kearns, 1993; Kearns and Mahanty, 1998). Therefore, the next logical step in the characterization of the AGA gene cluster was the determination of its boundaries.

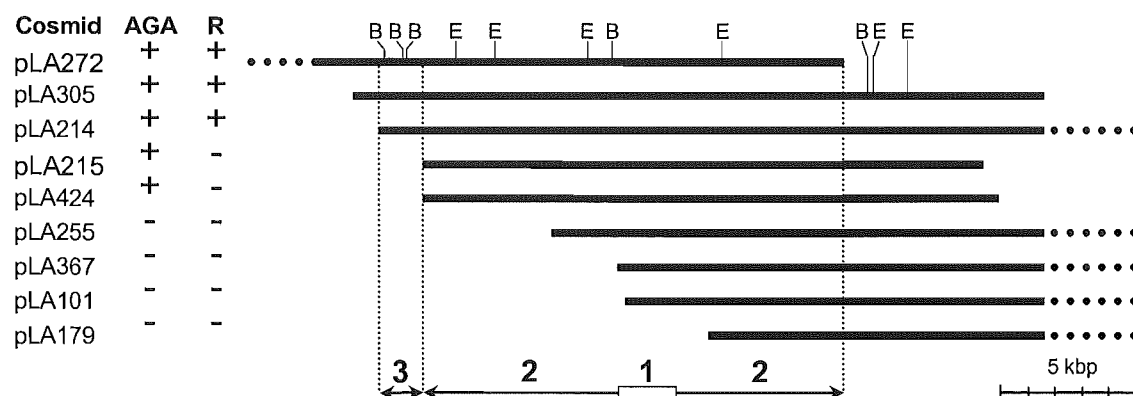


Figure 3.1. The DNA region required for AGA production and resistance.

Alignment of cosmids encoding all (+), or part (-), of the information required for AGA synthesis (AGA) and AGA resistance (R) in a heterologous host. Heavy dotted lines indicate that cosmid DNA extends beyond the region illustrated here. The recognition sites for *Bam*HI (B) and *Eco*RI (E) are shown. Arrows highlight: (1), the region of DNA in which *TnpH0A* insertions resulted in antibiotic-minus mutants (Kearns, 1993; Kearns and Mahanty, 1998); (2), the region common to cosmids that confer AGA production to a heterologous host; and (3), the region common to cosmids that confer AGA resistance on a heterologous host (discussed in section 3.8)

3.2.1. Left boundary of the AGA-gene cluster.

The search for the left-most boundary was initiated in a 7.4 kbp fragment of DNA bordered by *Bam*HI sites and located adjacent to region 1 (Fig. 3.1). To specifically manipulate this piece of DNA, it was transferred from pLA272 to pBR322, creating pBB7 (Fig. 3.2). Numerous mini-*Tn10*-LK transposon insertions were introduced into pBB7. Twelve were located within the 7.4 kbp *Bam*HI fragment, and four (U1 – U4), were transferred to the genome of Eh1087 by homologous recombination (Fig. 3.2). U1-U4 were chosen because they were located upstream of, and progressively further away from, the Eh1087 DNA cloned in cosmid pLA367 which does not confer AGA production on a heterologous host, and thus, could not possess the left-most boundary of the AGA gene cluster. U1 to U3 inactivated AGA-production in Eh1087 and demonstrated that information encoded in this region was required for antibiotic production. The insertion furthest from pLA367, U4, had no apparent effect on antibiotic production or

AGA-resistance. On this basis, U4 was assumed to lie outside the AGA-gene cluster and therefore defined the known left-most boundary.

3.2.2. Right boundary of the AGA-gene cluster.

The right-most boundary of the AGA-gene cluster was determined by a similar process, however this time DNA downstream of region 1 (depicted in Fig. 3.1) was disrupted by transposon insertions to determine which portion was required for antibiotic production. Initially, insertion D1 was isolated from a collection of pBE5a derivatives containing mini-Tn*phoA* insertions (pBE5a includes region 1). When introduced to the Eh1087 genome, insertion D1 created a mutant that was unable to produce antibiotic indicating that information further downstream of this point was involved in antibiotic production. During this investigation a 5 kbp *EcoRI* fragment of DNA from pLA272 that had been ligated to pBR322 to create pBE5b (Kearns, 1993) was found to lie adjacent to the DNA cloned in pBE5a (Fig. 3.2). Since all cosmids that confer AGA production on a heterologous host possess the DNA contained in pBE5b this DNA region was expected to contain information required for antibiotic production. Mutant derivatives of pBE5b were obtained by mutagenesis with mini-Tn*phoA*, and the position of five insertions determined. From these, insertions D2 and D3 were selected for transfer to the Eh1087 genome because they were located furthest from insertion D1. Insertion D2 interrupted antibiotic production, whereas an Eh1087 mutant with insertion D3 could still produce antibiotic (Fig. 3.2). This experiment demonstrated the region in which the AGA gene cluster terminated and thereby defined the extent of the AGA gene cluster.

3.2.3. The region between the proposed boundaries is sufficient for AGA production

To confirm that the region between the boundary insertions was sufficient for antibiotic production, the complete 15.5 kbp region was reconstructed, via a multi-step cloning process (data not shown), in pBluescriptKS- creating plasmid pAGA (Fig. 3.2D). pAGA was found to confer antibiotic production on *E. coli* hosts DH5 α and MC4100. These strains produced a moderate amount of antibiotic on minimal media, and a small amount on rich media (LB), demonstrating for the first time that the suppression of AGA production in *E. coli* by some component(s) of rich media is not absolute.

3.3. Isolation of Eh1087 AGA-minus mutants.

In order to identify all, or at least the majority of the genes involved in antibiotic production by Eh1087, new transposon mutants were generated, screened for loss of antibiotic production, and the genes disrupted in the AGA-minus mutants were characterized. A second mutant screen was deemed necessary for two reasons. Firstly, the original screen for Eh1087 mutants unable

to produce antibiotic was considered insufficient as discussed below (Kearns, 1993; Kearns and Mahanty, 1998). Secondly, antibiotic expression by *E. coli* strains harboring cosmids with the entire AGA-gene cluster (Fig. 3.1) was found to be repressed greatly in rich media in contrast to Eh1087 or other *Erwinia herbicola* isolates harboring these clones suggesting that additional 'Erwinia-specific' factors encoded outside the AGA gene cluster are required for full antibiotic production by Eh1087 in rich media.

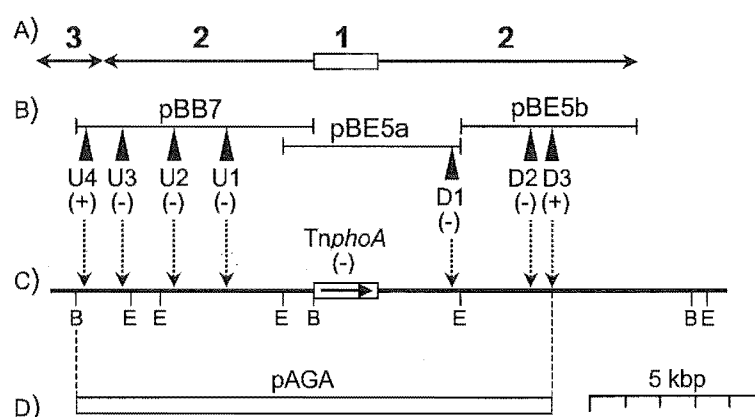


Figure 3.2. Creation of defined Eh1087 transposon insertion mutants

(A) The AGA-gene cluster regions described in Fig. 3.1

(B) A selection of transposon insertions into cosmid 3272 subclones pBB7, pBE5a, and pBE5b (see Table A1). The solid arrows indicate the positions of U1-U4 (insertions upstream of the *TnphoA* insertions) and D1-D3 (insertions downstream of the *TnphoA* insertions) as determined by restriction mapping.

(C) dashed arrows represent transfer of the transposon insertions to the genome of Eh1087 by homologous recombination to create Eh1087 derivatives that either cannot produce antibiotic (-) or retain the ability to produce antibiotic (+) and therefore define the AGA- gene cluster boundaries in Eh1087. *TnphoA* insertions created in a previous study cover a region of 2.2 kbp in which transcription was found to proceed left to right as illustrated by the horizontal arrow. (Kearns, 1993; Kearns and Mahanty, 1998). Recognition sites for *Bam*HI, B, and *Eco*RI, E, are shown for comparison with Figure 3.1. (D) The insert DNA for pAGA, covering the region between transposon insertions U4 and D3.

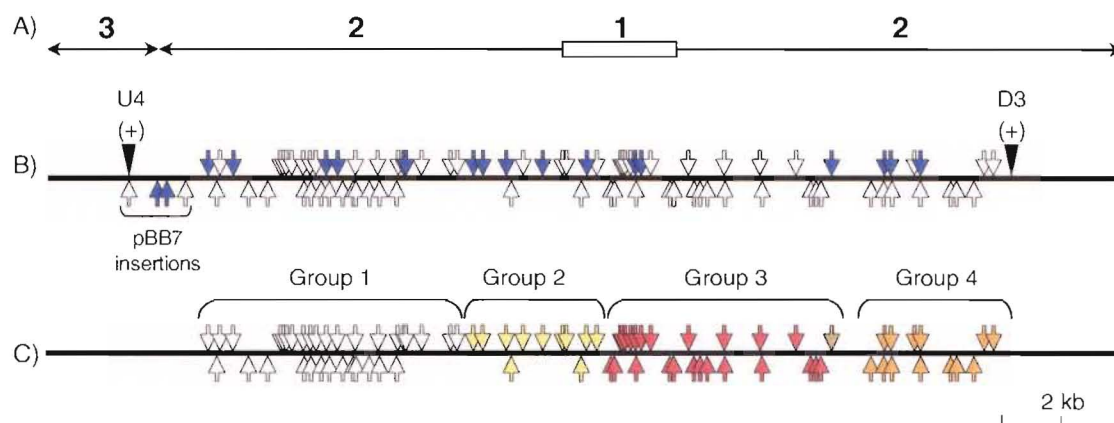


Figure 3.3. Positions of transposon insertions within the AGA gene cluster

(A) Regions 1, 2, and 3 are shown for comparison with Figure 3.1.

(B) The position and orientation of 82 mini-Tn5lacZ2 insertions that mapped within the AGA gene cluster of Eh1087 AGA-minus mutants, and of four mini-Tn10lacZ' insertions in and near the resistance gene encoded on plasmid pBB7. The positions of AGA+ insertions U4 and D3 are shown at each end of the gene cluster and delineate the gene cluster boundaries. Transposon insertions in orientation 1 are shown above the gene cluster, and insertions in orientation 2 below the gene cluster. Blue arrows represent insertions that are in the correct orientation and reading frame to produce β -galactosidase whilst white arrows represent insertions that do not produce β -galactosidase. The direction of transcription derived from these active lacZ fusions is generally from left to right, however insertions to pBB7 that result in loss of the AGA resistance factor demonstrate that the resistance gene is divergently transcribed from the rest of the AGA-gene cluster.

(C) Arrows are coloured to illustrate the colour of compounds produced by the AGA-minus mutants. Transposon insertions were divided into four groups based on the colour of the compound produced by each resulting mutant.

Initially, 1000 Eh1087 mini-Tn5lacZ2 mutants were screened to establish protocols for the assessment of antibiotic activity and for the mapping of transposon insertion points. Of the 1000 mutants screened, ten could not grow on minimal media. Since ca. 1% of the bacterial genome is generally dedicated to the metabolism of amino acids, the rate of auxotrophy found

here indicated that the mutagenesis screen was sufficiently random. A procedure to determine the positions of transposon insertions within the AGA gene cluster was developed to discriminate between insertions within the gene cluster and those outside it, and to map each insertion point within the cluster to 50-100 bp resolution (see materials and methods). A total of 5000 Eh1087 mutants were screened for the loss of antibiotic production, from which 83 mutants with single insertions were altered in their ability to produce antibiotic (1.7 %). Of these 83 mutants, 81 possessed insertions within the 14 kbp of AGA gene cluster (Fig. 3.3B), and 2 mutants contained insertions outside this region*. Insertions within the AGA gene cluster were distributed evenly throughout this region, in both possible orientations (Fig. 3B, Appendix A3).

3.4. Gene functions

3.4.1. Coloured AGA intermediates produced by many *ehp* mutants.

During the screening of Eh1087 mutants for loss of antibiotic production, a number of insertion points were cloned and the DNA sequence of the insertion points determined. A subset of these cloned insertion points was found to lie within a region of the Eh1087 genome with sequence similarity to genes involved in phenazine production by *Pseudomonas* spp., suggesting that the Eh1087 antibiotic is a phenazine antibiotic. Since some phenazine biosynthesis mutants of *Pseudomonas* spp. accumulate coloured phenazine precursor molecules when cultured on Kings-A medium, all 82 AGA-gene cluster mutants were cultured on this medium and assessed for the production of coloured compounds (Fig. 3.3C). Mutants with insertions in close proximity to each other usually produced compounds with similar colour, probably because the antibiotic biosynthesis pathway was inactive at the same modification step resulting in accumulation of the same-coloured antibiotic precursor. Intermediates of the same colour were assumed to have the same or very similar chemical structures and therefore used as a basis to divide the mutants into four groups (Fig. 3.3C). A yellow intermediate was named antibiotic precursor one (AP1), a red intermediate was named AP2, and an orange intermediate was named AP3. The colour of the intermediate(s) released by each mutant is listed in Appendix 3, and the properties of these intermediates are described in section 4.3.3.

* One of these mutants was found to have an insertion in *glnA*, which is responsible for the final step in glutamine synthesis (Appendix A2.4). The second mutant carries an insertion in an undefined gene that appears to be immediately upstream of a potential homologue of the *E. coli* sensor protein gene, *barA* (Appendix A2.5). Both mutants have subsequently become the focus of a separate investigation.

3.4.2. Precursor-feeding experiments.

To determine whether a mutant could convert an AGA precursor into AGA, isolated precursor compounds were provided exogenously to a representative mutant from each group (Fig. 3.4A). Griseoluteic acid (GA) was also assessed because it was a phenazine breakdown product of AGA present in the supernatant of Eh1087 cultures (as discussed in Chapter 4), its structure was known, and it was found to have similar properties to AP3 (see chapter 4). Only mutants with transposon insertions in orientation 1 (O1), and not those with orientation 2 (O2) insertions, are able to convert AP1, AP3, or GA into AGA. Figure 3.4A demonstrates that AP1 can be converted to AGA by Group 1 mutants, which cannot synthesize their own AP1 but can carry out all subsequent modifications. AP3 can be converted to AGA by Group 1, 2, and 3 mutants which are able to carry out the final conversion step, but not by Group 4 mutants, which produce AP3 but lack a gene required for conversion of AP3 to AGA. GA is also converted to AGA by Group 1, 2, and 3 mutants but not by Group 4 mutants, therefore the Group 4 gene products are required for the addition of a D-alanine group to GA to create AGA. It is worth noting that mutants representing insertions in each of the predicted gene cluster genes were subsequently assessed for precursor conversions and all mutants within a particular group possessed similar precursor conversion abilities (for example, see Figure 3.7); exceptions are discussed below.

3.4.3. Sharing of resources, AGA production by mixed *ehp* mutants

An alternative approach was required to investigate whether or not AP2 could be converted to AGA, because AP2 could not be separated from culture supernatant and concentrated by extraction with solvents such as chloroform or dichloromethane, unlike AP1, AP3 and AGA. Since Group 2 mutants release AP2 and Group 3 mutants release AP3, these mutants were used as donors of exogenous precursors. To do this, a selection of mutants representing transposon insertions in both orientations (O1 and O2) from each of the mutant groups was crossed pair-wise with each other mutant, as well as with the AGA gene-cluster deletion strain Eh Δ AGA. No individual mutant could complement Eh Δ AGA. In contrast, antibiotic production was observed when various combinations of mutants were crossed pair-wise (Fig. 3.4B). It was found that colonies composed of orientation 1 (O1) transposon insertion mutants from Group 4 and either Group 1, 2, or 3 produced antibiotic (Fig. 3.4B, plate 1). Similar antibiotic production was noted by colonies consisting of a Group 3 mutant and a Group 1 or Group 2 mutant. This result indicated that the gene Groups downstream of an O1 transposon insertion retained activity. In contrast, colonies composed solely of mutants with orientation 2 (O2) transposon insertions produced no antibiotic (Fig. 3.4B).

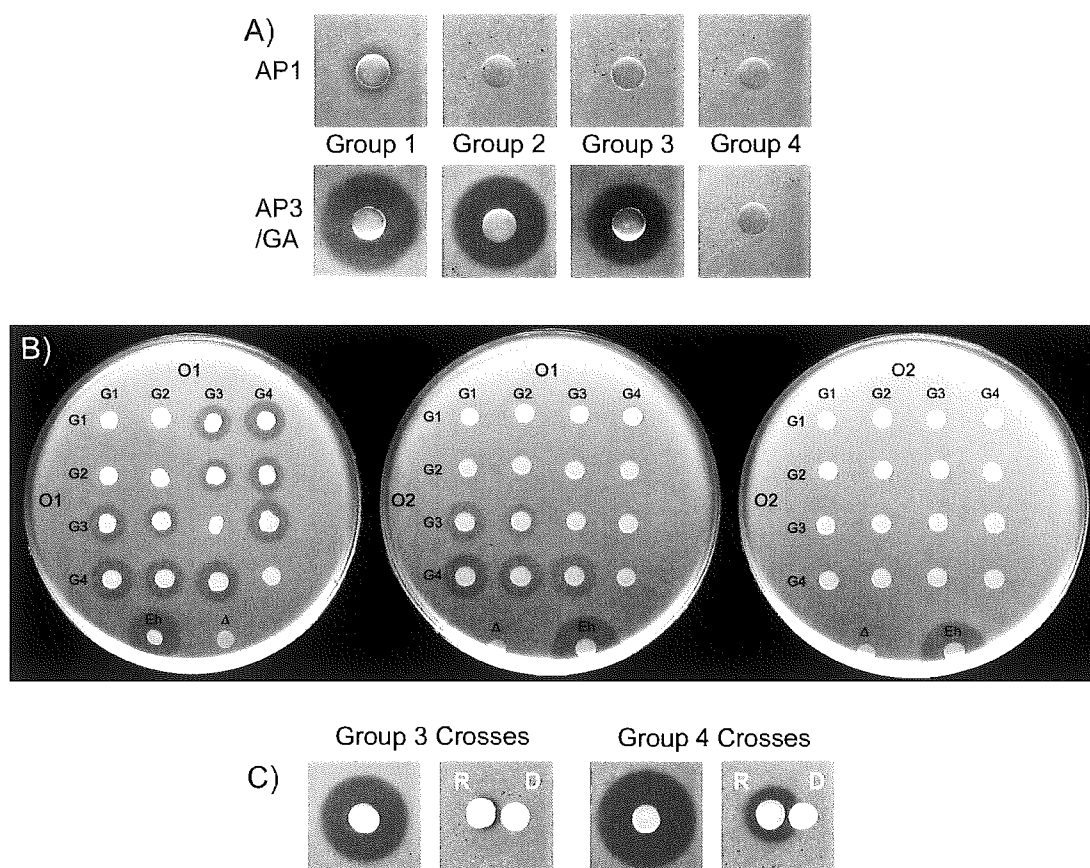


Figure 3.4. Conversion of intermediates to AGA by *ehf* mutants

(A) The conversion of exogenous AP1 and AP3 to AGA by mutants representative of each functional Group. Each six mm diameter filter disk contains 20 μ g of crude AP1 or AP3 and ca. 10^7 cells of the relevant mutant. In this diagram, antibiotic activity resulting from the conversion of AP1 or AP3 to AGA is demonstrated by a dark zone around a disk resulting from the death of Ea8862 cells spread over the plate (Griseoliteic acid (GA) gave the same result as AP3).

(B) Antibiotic production by colonies composed of two different *ehf* mutants. G1 (Group 1) – G4 (Group 4); O1, O2, transposon insertions in orientation 1 or 2.

(C) The relative conversion of AP2 and AP3 to AGA by mixed and separated colonies of *ehf* mutants. The Group 3 crosses are representative of a Group 3 mutant (AP2 donor, D) crossed with a Group 1 or Group 2 mutant (AP2 recipient, R), either as mixed, or separated colonies. Similarly, the Group 4 crosses are representative of crosses between a Group 4 mutant (AP3 donor) and a Group 1, 2, or 3 mutant (AP3 recipient).

To further assess the influence of transposon orientation on crossfeeding ability, O1 and O2 mutants were crossed pair-wise. O2 mutants only complemented O1 mutants that possessed insertions in a group upstream (relative to the direction of transcription throughout the AGA-gene cluster, as illustrated in Fig. 3.3). For instance, mutants with an O2 insertion in a Group 3 gene release AP2 (which is converted to AGA by a mutant with an O1 insertion in a Group 1 or 2 gene), but cannot convert AP2 or AP3 into AGA. Similarly, Group 1 or 2 mutants with an O2 insertion are not able to convert AP2 or AP3, donated by O1 mutants, into AGA. These results concur with the inability of the O2 mutants to convert exogenous AP1, AP3, or GA, to antibiotic, as described in section 3.4.3, and indicate that all genes downstream of an O2 transposon insertion are either not expressed, or expressed at a far lower level than normal.

To confirm the mutant responsible for donation and the mutant responsible for precursor conversion, colonies of each mutant were separated by ca. 1-2 mm and assessed for antibiotic production (Fig. 3.4C). This experiment demonstrated that AP3 is donated by Group 4 mutants, and converted to AGA by Group 1, 2 or 3 (O1) mutants which must therefore produce functional Group 4 gene products to carry out this conversion. Similarly, Group 3 mutants were shown to donate AP2 under the same conditions to Group 1, 2, or 3 (O1) mutants, which express functional Group 4 gene products and can convert AP3 to AGA. Colonies receiving exogenous AP3 were able to convert it to AGA at a faster rate than those receiving exogenous AP2. Because the rate of conversion of the two precursors was similar in mixed colonies, and since the red-coloured AP2 diffuses through the agar media at least as well as AP3, it appears that the transfer of AP2 from one mutant to another is facilitated by cell-to-cell contact, whereas the equivalent transfer of AP3 is less dependent on such contact. The low rate of AP2 to AGA conversion by adjacent colonies of mutants meant that this experiment could not demonstrate whether Group 4 mutants can convert AP2 to AGA, however this is improbable because Group 4 mutants lack one of the genes required for the conversion of AP3 to AGA.

3.5. Description of the AGA-gene cluster genes

3.5.1. DNA sequence of the AGA gene cluster.

Mutants with transposon insertions in the AGA-gene cluster could be divided into Groups depending on the colour of the AGA intermediate(s) that they produced (Fig. 3.3C). To begin characterizing the number and function(s) of genes within these groups the DNA sequence of the entire region between insertions U4 and D3 was determined and assessed for sequence determinants characteristic of genes using the GeneMark algorithm (Lukashin and Borodovsky,

1998). From the 15497 bp of sequence data analyzed, GeneMark predicted 16 genes within a contiguous 14924 bp section of the DNA sequence, designated *ehpR*, and *ehpA-ehpO* (*Erwinia herbicola* phenazine) (Fig. 3.5, Table 3.1). There are likely to be 5 genes, named *ehpG-ehpK* present in the 5061 bp region of DNA previously reported to contain 6 ORFs (Kearns and Mahanty, 1998). *ehpI*, *ehpJ*, and *ehpK* were formerly designated ORFs 4, 5, and 6, respectively, while *ehpG*, and *ehpH* are new predictions arising from the change in sequence context. Most importantly, the predicted genes agreed well with the division of mutants into groups, as reported in Appendix A3.

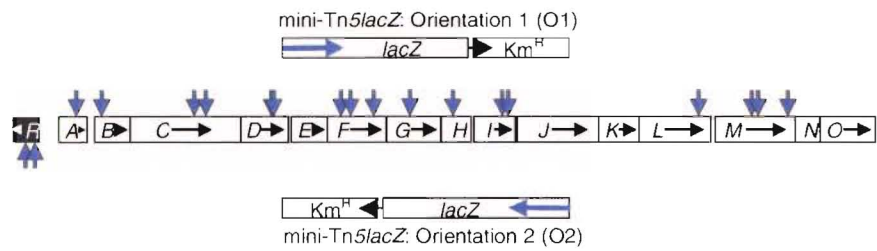


Figure 3.5. The *ehp* gene cluster

Location, size, and direction of transcription of the *ehp* genes predicted by GeneMark analysis of the DNA sequence between insertions U4 and D3. Blue arrows indicate the position of in-frame *lacZ* fusions.

3.5.2. Evidence in support of the *ehp* gene predictions

Three lines of experimental evidence support the predicted properties of the genes illustrated in Figure 3.5. Firstly, mutants with transposon insertions in the same sequence-predicted gene excrete the same coloured compounds when grown on Kings-A media (listed in Appendix A3). Secondly, the predicted direction of transcription for each of the genes concurs with the orientation of active *lacZ* fusions to the *ehp* genes A, B, C, D, F, G, H, I, L, and M (Fig. 3.5). Finally, the sizes of many of the predicted translation products of the *ehp* genes were confirmed by minicell analysis of these gene products as outlined below.

E. coli minicell strain P678-54 was transformed with one of three plasmids that collectively contain all the DNA of the AGA gene cluster, pBB7, pBE5a or pBE5b, or derivatives of these plasmids into which mini-Tn5*lacZ2* insertions had been transferred (see methods and materials, Table 3.1). To relate a single gene product to a transposon insertion, O1 insertions were used to ensure that genes downstream of the insertion point were expressed via initiation of transcription for the kanamycin resistance gene of mini-Tn5*lacZ2*, which does not possess transcriptional or translational stop signals (de Lorenzo et al., 1990).

E. coli minicells containing pBB7 produced radiolabelled EhpR (15 kDa), EhpA (19 kDa), EhpB (26 kDa), EhpD (32 kDa), EhpE (28 kDa) and EhpF (48, 52 kDa) (Fig. 3.6). In most cases these products are similar in size to the predicted gene product (Table 3.1). A range of minicell strains containing pBB7 derivatives with an O1 transposon insertion in an *ehp* gene demonstrated the size of each gene product (Fig. 3.6). The products of *ehpB* and *ehpE* are slightly different in size to the sequence predictions, whilst insertion to *ehpF* resulted in the loss of 2 proteins, both larger than predicted from sequence data, suggesting that these proteins may undergo post-translational modifications if the sequence predictions are accurate. It is unknown why insertion to *ehpC*, which has a predicted 70 kDa product, did not result in the disappearance of any detectable proteins. *E. coli* minicells containing pBE5a and pBE5a::*ehpI* demonstrated clear products for EhpI (26 kDa) and EhpK (29 kDa), but not for EhpG (33 kDa), EhpH (17.5 kDa) or EhpJ (55 kDa) (Fig. 3.6). The three proteins not detected by this procedure are thought to be bound to the inner membrane as described in sections 3.6.2 and 3.6.3. As such they are likely to be hydrophobic and may have not have dissolved in the protein loading buffer prior to electrophoresis. *E. coli* minicells containing pBE5b and pBE5b::*ehpM* demonstrated a clear product for EhpM (56 kDa), and a second 12 kDa protein that may EhpN, however this was not confirmed with an *ehpN* knockout (Fig. 3.6). No EhpO candidate protein was seen, probably because the predicted 34 kDa protein was concealed by a highly expressed protein that is probably the kanamycin resistance protein (31 kDa) since it is present in cells containing only the plasmid vector. Finally, EhpL was not observed in these analyses because *ehpL* spans between pBE5a and pBE5b.

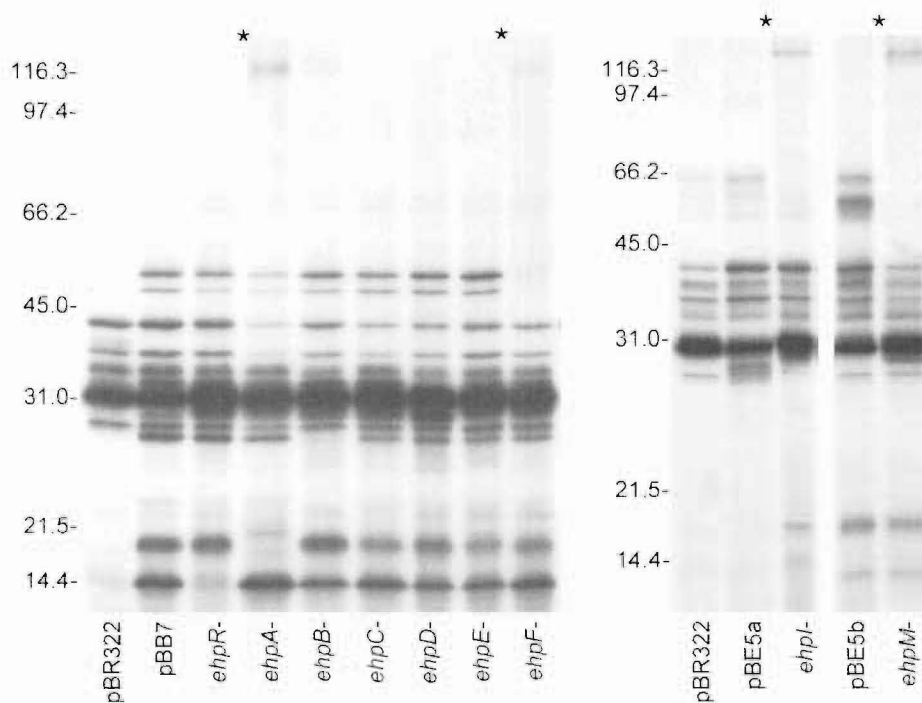


Figure 3.6. Minicell analysis of gene cluster protein products

(A) Proteins from minicells harbouring pBB7 and various gene-knockout derivatives were resolved by SDS-PAGE in a 12 % linear gel.

(B) Proteins from minicells harbouring pBE5a, pBE5a::*ehpI*, pBE5b, pBE5b::*ehpM* were resolved by SDS-PAGE in a 8 % - 16 % gradient gel. Bands to the right of an asterisk are presumably LacZ::Ehp fusion proteins.

3.6. Gene functions derived from sequence predictions and experimental evidence

A BlastN search for similarity between the DNA sequence of the *ehp* gene cluster and other reported DNA sequences indicated that the DNA sequences of the *ehp* genes are novel (Altschul et al., 1997). However, as discussed below, BlastX demonstrated that the translation products of some genes within the cluster were similar to the predicted products of genes from various sources. In addition, the predicted sequences of the Ehp proteins were assessed for conserved domains using RPS-BLAST, for signal sequences using SignalP, and for membrane associated regions using DAS and TMHMM, as outlined in Appendix A1.7.2 (Table 3.2).

Table 3.1. Properties of the *ehp* genes

¹ Group	(Colour)	² Intermediate	Gene	³ Position	⁴ AA	⁵ kDa	(⁶ est.)
-	-	-	<i>ehpR</i>	918-529	129	14.6	(15)
G1	(none)	-	<i>ehpA</i>	1337-1813	159	18.6	(19)
G1	(none)	-	<i>ehpB</i>	1944-2576	261	29.4	(26)
G1	(none)	-	<i>ehpC</i>	2644-4473	635	70.2	
G1	(none)	-	<i>ehpD</i>	4473-5336	288	31.8	(32)
G1	(none)	-	<i>ehpE</i>	5470-6000	209	23.7	(28)
G2	(yellow)	AP1	<i>ehpF</i>	5981-7081	367	40.9	(48,52)
G2	(yellow)	AP1	<i>ehpG</i>	7081-8016	312	33.3	
G2	(yellow)	AP1	<i>ehpH</i>	8013-8489	159	17.7	
G3	(red)	AP2	<i>ehpI</i>	8534-9217	228	25.0	(26)
G3	(red)	AP2	<i>ehpJ</i>	9241-10677	479	50.8	
G3	(red)	AP2	<i>ehpK</i>	10677-11465	263	29.3	(29)
G3	(red)	AP2	<i>ehpL</i>	11476-12612	379	40.3	
G4	(orange)	AP3	<i>ehpM</i>	12683-14164	494	55.9	(56)
G4	(orange)	AP3	<i>ehpN</i>	14154-14381	76	8.5	(12)
G4	(orange)	AP3	<i>ehpO</i>	14352-15452	310	34.0	

1. Mutant groups derived from the production of coloured AGA intermediates
2. AGA intermediates AP1, AP2 and AP3 were differentiated on the basis of colour, and could consist of a group of similarly coloured compounds
3. Position of each predicted *ehp* gene (including start and stop codons) within the 15524 bp AGA-gene cluster DNA sequence between transposon insertions U4 and D3.
4. Number of amino acid residues in the predicted translation product of each *ehp* gene.
5. The predicted molecular weight of the *ehp* gene products in kilodaltons
6. The estimated molecular weight of Ehp proteins derived from minicell analysis of the AGA-gene products where known.

3.6.1. The Group 1 genes products.

Two lines of evidence indicate that the group 1 genes are responsible for the synthesis of AP1. Firstly, exogenously supplied AP1 partially restores antibiotic production to Group 1 mutants, demonstrating that these mutants lack the ability to synthesize AP1 but possess the capability of subsequent conversion of AP1 into AGA (Figure 3.7). Secondly, AP1 is produced by cells harbouring the Group 1 genes but no other *ehp* genes. The predicted protein sequences of the Group 1 genes, *ehpA-ehpE*, show many identities to the products of five out of seven genes that

form a core operon required for synthesis of phenazine-1-carboxylic acid (PCA) by *Pseudomonas* spp. (Table 3.1). The arrangement of the phenazine synthesis genes in the PCA operons is also similar to the Group 1 genes (Fig. 3.8A), however in Eh1087 the Group 1 gene products synthesize AP1, a probable phenazine compound that was shown by HPLC analysis to have a similar absorbance spectrum, but different solubility in water than PCA (see Fig. 4.7).

Table 3.2. Properties of the predicted *ehp* translation products

Product	Role	Domain ¹	Residues ²	Aligned ³	Score ⁴	TM ⁵
EhpR	AGA ^R	none				No
EhpA	→AP1	none				No
EhpB	→AP1	Isochorismatase family	27-207 (261)	97.8%	4e ⁻⁴⁶	No
EhpC ^a	→AP1	Chorismate binding enzyme	112-379 (635)	100.0%	4e ⁻³⁹	No
		Glutamine amidotransferase class-I	441-621 (635)	98.4%	1e ⁻¹⁷	
EhpD	→AP1	Phenazine biosynthesis-like protein	6-277 (288)	98.0%	2e ⁻³⁴	No
EhpE	→AP1	Pyridoxamine 5'-phosphate oxidase	5-208 (209)	93.2%	7e ⁻³⁵	No
EhpF	AP1→AP2	AMP-binding enzyme	44-315 (367)	60.5%	9e ⁻⁰⁷	No
EhpG	AP1→AP2	Aldehyde dehydrogenase family	155-294 (312)	32.0%	4e ⁻⁰⁸	1*(D)
EhpH	AP1→AP2	none				2 (D)
EhpI	AP2→AP3	ubiE/COQ5 methyltransferase family	51-214 (228)	70.5%	3e ⁻⁰⁷	No
EhpJ ^b	AP2→AP3	none				14*(T)
EhpK	AP2→AP3	Short chain dehydrogenase	6-223 (263)	90.0%	2e ⁻²⁵	No*
EhpL	AP2→AP3	none				No
EhpM	AP3→AGA	AMP-binding enzyme	42-420 (494)	100.0%	1e ⁻⁵²	1 (T)
EhpN ^b	AP3→AGA	none				No
EhpO	AP3→AGA	none				No

1. Conserved domains of the predicted *ehp* translation products detected by RPS-BLAST. Note: a) EhpC has two domains, b) PROSITE detected motifs for EhpJ (transmembrane transport protein), and EhpN (acyl carrier protein domain) that were not detected by RPS-BLAST).
2. Residues within the Ehp protein that comprise the conserved domain. The total size of the Ehp protein is shown in brackets for comparison.
3. Percentage of the conserved domain present in the Ehp protein.
4. Significance scores for the likelihood that each sequence similarity or conserved domain occurred by chance (only proteins with scores above 1e⁻⁶ are shown).
5. TM, transmembrane sections predicted by DAS (D) with a cutoff value of 2.2, or by TMHMM (T); an asterisk indicates that the protein is predicted to have a prokaryotic membrane lipoprotein lipid attachment site

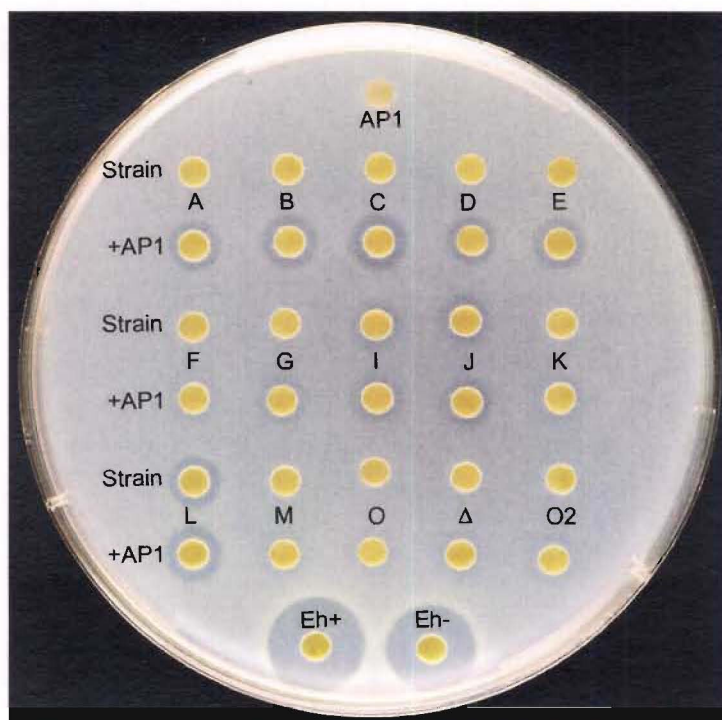


Figure 3.7. AP1 conversion by representative *ehp* mutants

Six mm filter disks with 20 μ g AP1 (+AP1) and without AP1 (negative control) were supplemented with 10^7 cells of each *Ehehp* mutant and assessed for antibiotic activity on a lawn of 1a8862 grown on a 1-A minimal media agar plate. A disk with AP1 only, or with AP1 and the *ehp* gene cluster deletion strain *Eh* Δ AGA (Δ), show no antibiotic activity in this assay. All mutants possess O1 transposon insertions, and an O2 *ehpA* mutant (O2) is shown for comparison. *Eh*1087 supplemented with 20 μ g AP1 (*Eh*+) produces slightly more antibiotic than non-supplemented *Eh*1087 (*Eh*-).

The predicted translation products of the Group I genes *ehpB-ehpE* possess conserved domains that concur with the predicted functions of the PCA operon analogs (Fig. 3.8, Table 3.2). The sequence of *EhpB* indicates that it belongs to the isochorismatase family of enzymes, of which it is most similar to the PhzA-PhzD proteins required for phenazine synthesis in *Pseudomonas*, and next most similar to anthranilate synthetase which catalyzes the exchange of the amide nitrogen of glutamine for the side group of chorismic acid converting it into anthranilate. The predicted sequence of *EhpC* aligns closely to the PhzB-PhzE phenazine synthesis proteins. PhzC possesses two domains, one is characteristic of chorismate binding enzymes, and the other is a member of the glutamine amidotransferase class-I family. *EhpD* shares sequence similarity

with PhzC-PhzF. A recently report by McDonald *et al.* (2001) indicates that PhzF acts with PhzG in the dimerization of two molecules of 2,3-dihydro-3-oxoanthranilic acid (DHOA) to form PCA. Ehpd may possess two DAP-epimerase motifs (weak similarity), which concur with the prediction that the *Pseudomonas* analog, PhzF, holds two DHOA molecules in the correct orientation to control dimerization. Finally, Ehpe is similar to PhzG, and both have sequence characteristics of pyridoxamine 5'-phosphate oxidases. This domain is predicted to enable PhzG to oxidize trans-2,3-dihydro-3-hydroxyanthranilic acid (DHHA) to DHOA as part of the dimerization process (McDonald *et al.*, 2001). By comparison with the *Pseudomonas* model (Fig 3.8B), Ehpc is expected to transfer an amide group from glutamine to chorismic acid, while EhpB removes a pyruvate moiety to create DHHA. DHHA is most likely converted to a molecule very similar to PCA by EhpD and Ehpe, and EhpA may help to stabilize a hypothetical multienzyme complex and thereby enhance the rate of phenazine synthesis (Mavrodi *et al.*, 1998).

3.6.2. The Group 2 gene products.

Group 2 mutants are unable to convert AP1 to AP2 (Figs. 3.4, 3.7). The DNA and predicted protein sequences of the Group 2 genes did not show similarity to the sequences of genes or gene products with known function(s). Therefore, to begin characterizing each gene involved in the conversion of AP1 to AP2, the DNA and translated protein sequences of the Group 2 genes were analyzed for conserved functional domains or motifs using RPS-BLAST and PROSITE. The protein sequence between residues 72-124 of EhpF has a weak similarity to ca. 60% of a conserved domain characteristic of AMP-binding proteins. EhpG has a weak similarity to the middle ca. 30% of a conserved domain found in aldehyde dehydrogenases. EhpG and EhpH both possess features that suggest these proteins are localized to the cytoplasmic membrane. Specifically, residues 275-285 of EhpG are characteristic of a prokaryotic membrane lipoprotein lipid attachment site, for which a signal peptide at the C-terminus is cleaved by signal peptidase II and a glyceride-fatty acid lipid is bound to a cysteine residue for membrane attachment. This prediction is strengthened by the analysis of EhpG with the Dense Alignment Surface method (DAS) (Cserzo *et al.*, 1997) which indicates that EhpG has a single, potentially membrane-bound, section located between residues 274-285. Similarly, the DAS method predicted that EhpH has two hydrophobic regions, between residues 68-76 and 86-96, that could interact with a membrane. While the mechanism for conversion of AP1 to AP2 is currently unknown, the potential for membrane interactions by EhpG and H suggests that it takes place on or near the cytoplasmic membrane.

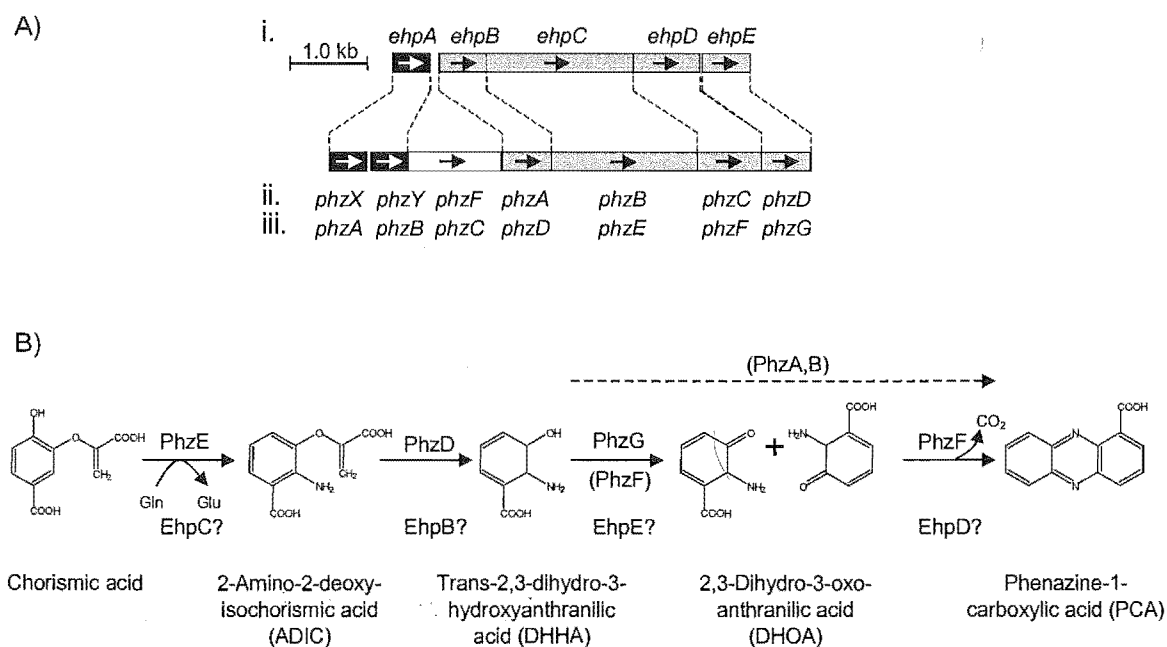


Figure 3.8. Analysis of the Group 1 genes

(A) Comparison of genes required for synthesis of the phenazine nucleus in:

- (i) Eh1087
- (ii) *P. aureofaciens* 30-84
- (iii) *P. chlororaphis* PCL1391, *P. fluorescens* 2-79, and *P. aeruginosa* PAO1
(PAO1 possesses two *phz* operons, *phzA1-phzG1*, and *phzA2-phzG2*).

The black boxes highlight the similarity between the translation product of *ehpA* and the translation products of both *phzX* and *phzY* (or *phzA* and *phzB*). The white box highlights the *phzC*/*phzF* gene specific to the phenazine gene clusters found in *Pseudomonas* spp., and grey boxes indicate genes from each bacteria that have similar translation products and are in the same relative location.

(B) The predicted role of the PCA operon gene products (Phz) in the synthesis of the phenazine nucleus by *P. fluorescens* 2-79 (adapted from McDonald et al., 2001), and the probable roles of Ehp analogs from Eh1087. Phz proteins in brackets are not essential, however PhzF may act with PhzG in the oxidation of DHHA, and PhzA and PhzB are known to increase the efficiency of the conversion of DHHA to PCA. The conversion of all intermediates to phenazine has been demonstrated with the exception of 2,3-dihydro-3-oxoanthranilic acid (DHOA).

3.6.3. The Group 3 gene products.

The Group 3 genes are required for conversion of AP2 to AP3 (Fig. 3.4, Table 3.2). Analysis of the protein sequence of the predicted translation products of the Group 3 genes provided information about the activities of EhpI, EhpJ, and EhpK, but not about EhpL. EhpI contains ca. 70% of a methyltransferase motif between residues 51-215, which may direct the transfer of a methyl group from S-adenosyl-L-methionine to the antibiotic precursor AP2. Because no other AGA-gene products appear to have this motif EhpI is currently the best candidate to catalyze the addition of the methoxy group present in AGA (see Fig. 4.6). EhpK may also be involved in direct modification of AP2 because it has a protein sequence containing 90 % of a motif characteristic of short chain dehydrogenases, a family that includes NAD- and NADP-dependent oxidoreductases.

The predicted protein sequence of EhpJ is similar to transmembrane efflux proteins found in a variety of bacterial species. PROSITE analysis of the EhpJ sequence indicates that it belongs to a group of proteins involved in the transmembrane transport of various compounds including sugar transporters, the FecCD transport family, and the Xanthine / Uracil permease family. Support for the transmembrane localization of EhpJ is provided by the predicted presence of a region rich in the hydrophobic amino acid leucine (residues 209-288), and the prediction by the transmembrane hidden Markov model (TMHMM) that EhpJ has 14 transmembrane sections.

To establish the EhpJ membrane topology experimentally, random mini-Tn5*phoA* fusions were generated throughout clone pBE5a, which contains the genes *ehpG-ehpK*. Active *PhoA* fusions indicate fusions to part of a protein that is localized extracytoplasmically because alkaline phosphatase is not active in the cytoplasm (Michaelis et al., 1983). Active fusions only originated from insertions within *ehpJ*, suggesting that EhpJ does indeed have periplasmic domains, and that the products of the other *ehp* genes encoded by pBE5a are located in the cytoplasm. The exact location of a selection of active mini-Tn5*phoA* insertions in *ehpJ* was determined by DNA sequence analysis. Five different insertions were obtained. The five hybrid proteins predicted to result from these mini-Tn5*phoA* insertions were composed of a fusion between *PhoA* and 21, 88, 148, 221, or 271 amino acids from the N-terminus of EhpJ, confirming the first five out of seven predicted periplasmic domains (Fig. 3.9). TMHMM-analysis of EhpJ predicted that for each of these hybrid proteins *PhoA* would be fused to a section of EhpJ that either transverses from the inner to outer sides of the membrane, or is located on the periplasmic side of the membrane. Analysis of the amino acid sequence of EhpJ with SignalP indicates that the first transmembrane domain may be a N-terminal signal peptide that is cleaved between residues 20-21 or 43-44, resulting in a mature EhpJ protein with 12 or

13 transmembrane domains. Because PhoA::EhpJ fusion data agree with analyses of the predicted protein sequence of EhpJ, it is likely that EhpJ is a transmembrane protein.

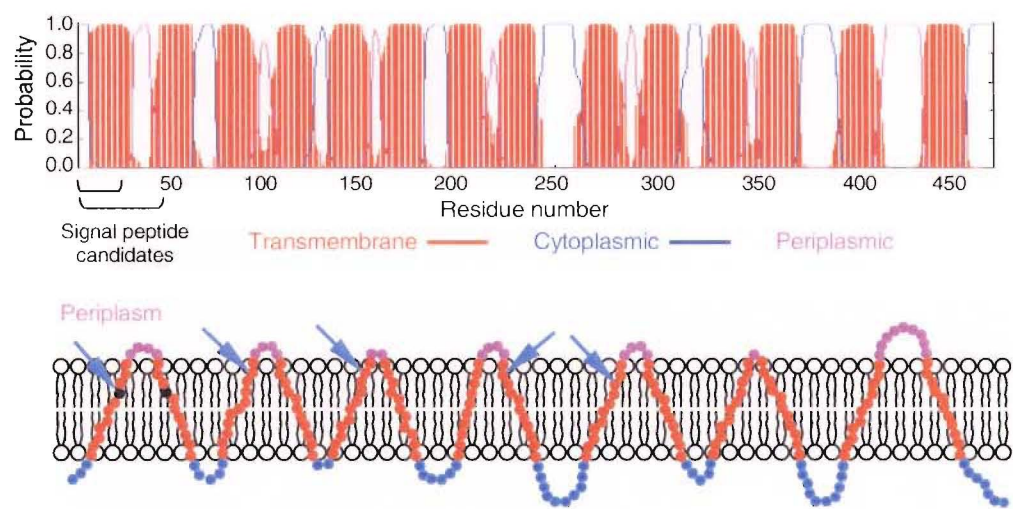


Figure 3.9. Predicted membrane topology for EhpJ

The TMHMM-predicted probability of regions of EhpJ protein to be cytoplasmic, periplasmic or membrane-localized is shown in the top section of the illustration, and the proposed membrane localization is shown below. Each coloured dot represents two amino acids. Black dots indicate the positions of potential signal sequence cleavage points, and blue arrows show the position of active PhoA fusions.

Comparison with other transport proteins suggest that the role of EhpJ in the conversion of AP2 to AP3 is likely to involve controlled transport across the cytoplasmic membrane. AP2 is only released by Group 3 mutants presumably due to lack of a transmembrane complex required for controlled release. Group 4 mutants or Eh1087 do not release AP2, as evidenced by the absence of red compounds in the culture media of the latter strains after most AP3 and AGA was removed by numerous chloroform extractions, indicating that AP2 is always converted to AP3 and released by cells with functional Group 3 genes.

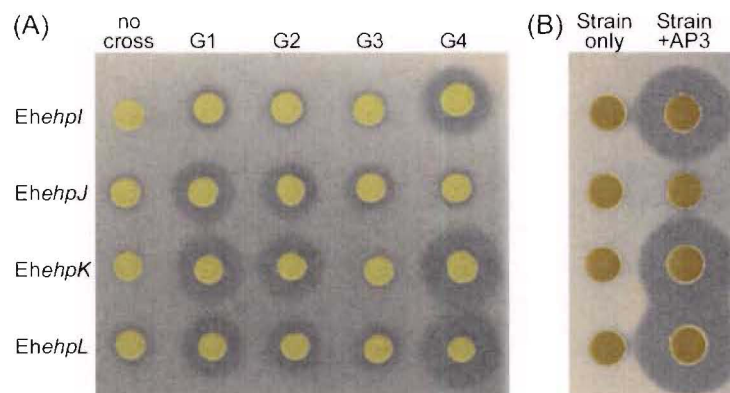


Figure 3.10. Properties of the Group 3 mutants

- (A) The conversion of AP2 donated by O1 Group 3 mutants to AGA by representative O1 Group 1 – Group 4 mutants (G1 – G4).
- (B) The conversion of exogenous AP3 to AGA by Group 3 mutants. Each 6 mm filter disk contains ca. 5×10^7 CFU of *EhehpI*, *J*, *K* or *L* with or without 10 μ g AP3.

A closer inspection of AGA production by O1 and O2 Group 3 mutants crossed with representatives from other groups enabled the Group 3 genes to be further subdivided according to the specific role of each gene product in the conversion of AP2 to AP3 (Fig. 3.10). All Group 3 mutants export a red coloured compound, designated AP2, when cultured in Kings-A media, with the exception of *EhehpL* (O1), which releases a red / brown compound. The red compound released by *EhehpI* (O1) is taken up and converted to AGA by Group 1 or Group 2 mutants far less proficiently than the red compound(s) released by all other Group 3 mutants (Fig. 3.10). On the basis of this and other properties (listed in Table 3.3), the red compound (AP2^{II}) produced by *EhehpI* (O1) is clearly different from the red compounds produced by other mutants (AP2 / AP2^I). Furthermore, since there is a good chance that Ehpl catalyses the addition of a methyl group to AP2, the red compound produced by mutants expressing Ehpl was tentatively termed AP2^I, to differentiate it from the red compound released by *EhehpI* (O2) which lacks Ehpl and all other Group 3 gene products. However, there is no experimental evidence to indicate that AP2 and AP2^I are different at this stage. In summary, different Group 3 mutants produce one of at least two different red compounds; *EhehpI* (O1) produces AP2^{II}, and all other Group 3 mutants produce AP2 / AP2^I. Important properties of AP2 / AP2^I and AP2^{II} are listed in Table 3.3.

Table 3.3. Origin and properties of coloured compounds produced by Group 3 mutants.

Insertion ¹		Activity of the Group 3 genes ²				Media colour ³		Convert. ⁴	Name ⁵
Gene	Ori.	<i>ehpI</i>	<i>ehpJ</i>	<i>ehpK</i>	<i>ehpL</i>	sealed	opened		
<i>ehpI</i>	O2	off	off	off*	off*	red	yellow	yes	AP2
<i>ehpJ</i>	O2	on	off	off*	off*	red	yellow	yes	AP2 ^I
<i>ehpK</i>	O2	on	on	off	off	red	yellow	yes	AP2 ^I
<i>ehpL</i>	O2	on	on	on	off	red	yellow	yes	AP2 ^I
<i>ehpI</i>	O1	off	on	on	on	red	red	no	AP2 ^{II}
<i>ehpJ</i>	O1	on	off	on	on	red	yellow	yes	AP2 ^I
<i>ehpK</i>	O1	on	on	off	on	red	yellow	yes	AP2 ^I
<i>ehpL</i>	O1	on	on	on	off	brown	brown	yes	AP2 ^{I?}

1. The interrupted Group 3 gene and the orientation (Ori.) of mini-Tn5*lacZ2* within that gene.
2. Activity of the Group 3 genes predicted from transposon orientation and demonstrated by DNA complementation (see section 3.4). **ehpK* and *ehpL* are expressed at a low level when *ehpI* or *ehpJ* are interrupted (see section 3.7).
3. The colour of compound(s) excreted to Kings-A media by each mutant cultured in sealed (oxygen limited) or opened (unlimited oxygen) microtitre dishes. The colours present in sealed microtitre dishes changed from red to yellow within hours of opening.
4. Shown to be converted (Convert.) into antibiotic by Group 1 and 2 mutants (a measure of the activity of Group 3 and Group 4 genes).
5. The division of red compounds according to different properties. The brown compound (AP2^{I?}) produced by *EhehpL* (O1) presumably arises from mini-Tn5*lacZ2*-induced expression of the neighbouring Group 4 gene *ehpM*, as the equivalent O2 mutant, *EhehpL* (O2), produces a red compound.

Since AP2^{II} is only released by a mutant expressing *ehpJ*, *ehpK*, and *ehpL*, but not *ehpI*, it is apparent that one or more of the proteins EhpJ, EhpK, and EhpL (EhpJKL), convert AP2 into a form that either cannot be taken up by a Group 1 or 2 mutant or cannot be further modified into AGA by the Group 3 and Group 4 gene products. Either way, EhpJKL can modify AP2. Whenever EhpI is present, or when all Group 3 gene products are missing a red compound is released that can be modified to AGA. Therefore, EhpI is either inactive in the absence of EhpJ-L and AP2 is the same as AP2^I, or EhpI modifies AP2 in such a way that it can still be converted to AGA, in which case AP2 and AP2^I would be different. Sequence analysis suggests that EhpI is a methyltransferase and EhpK is an oxidoreductase, thus EhpI and EhpJKL probably catalyze different reactions. Interestingly, it was noted that the red compound AP2 / AP2^I released by the appropriate mutants grown in sealed Universal bottles became

yellow/brown once opened, in contrast to AP2^{II}. Once the bottles were resealed the culture media again became red after many hours, and the conversion from yellow to red started at the bottom of the culture medium. These observations indicate that exposure to some component in air, presumably oxygen, is in part responsible for the colour change of AP2 / AP2^I. It is likely that AP2^{II} is not susceptible to these changes because it has been modified by the EhpJKL complex, of which EhpK is a potential oxidoreductase that may alter the redox state, or at least the oxygen-reactivity, of AP2.

Figure 3.10 also illustrates how *EhehpJ* (O1) cannot convert exogenous AP3 to AGA, in contrast to all other Group 3 mutants. This observation enables at least one function of EhpJ to be differentiated from EhpK and EhpL. As described section 3.7.4, the Group 4 genes *ehpM*, *ehpN*, and *ehpO*, cloned in pBR322 to create pBE5b, confer on a host bacterium the ability to convert AP3 to AGA in the absence of all other *ehp* genes, including *ehpJ*. The Group 4 genes in pBE5b are orientated so that they are likely to be expressed under control of the promoter of the Tc^R gene present in the vector, pBR322, probably overriding the normal regulatory mechanisms that control expression of the Group 4 genes in Eh1087. Therefore, the Group 4 gene products do not need to interact with EhpJ to function. However, in the context of the complete *ehp* gene cluster, *ehpJ* must not be interrupted for full expression of the Group 4 genes. Given that O2 *ehpK* and *ehpL* mutations do not affect expression of the Group 4 genes the influence of *ehpJ* is unlikely to be *cis*-acting, and therefore probably involves a *trans*-acting mechanism.

A diagram of the predicted activities of the Group 3 gene products in different Group 3 mutants is presented in Figure 3.11. The complete Group 3 system present in Eh1087 (Panel 1), converts AP2 to AP3, and releases AP3 on the periplasmic side of the inner membrane. Panel 2 illustrates the release of AP2 by *EhehpI* (O2), a mutant which effectively lacks all Group 3 gene products. Panel 3 shows the release of AP2^{II} by *EhehpI* (O1), a mutant that lacks EhpI, but possesses EhpJ, EhpK, and EhpL, and modifies AP2 to AP2^{II}. Panels 4, 5, and 6 illustrate strains in which EhpI is present and AP2^I is released. Determination of the structures of the red compounds released by different Group 3 mutants would strongly support or discredit this model.

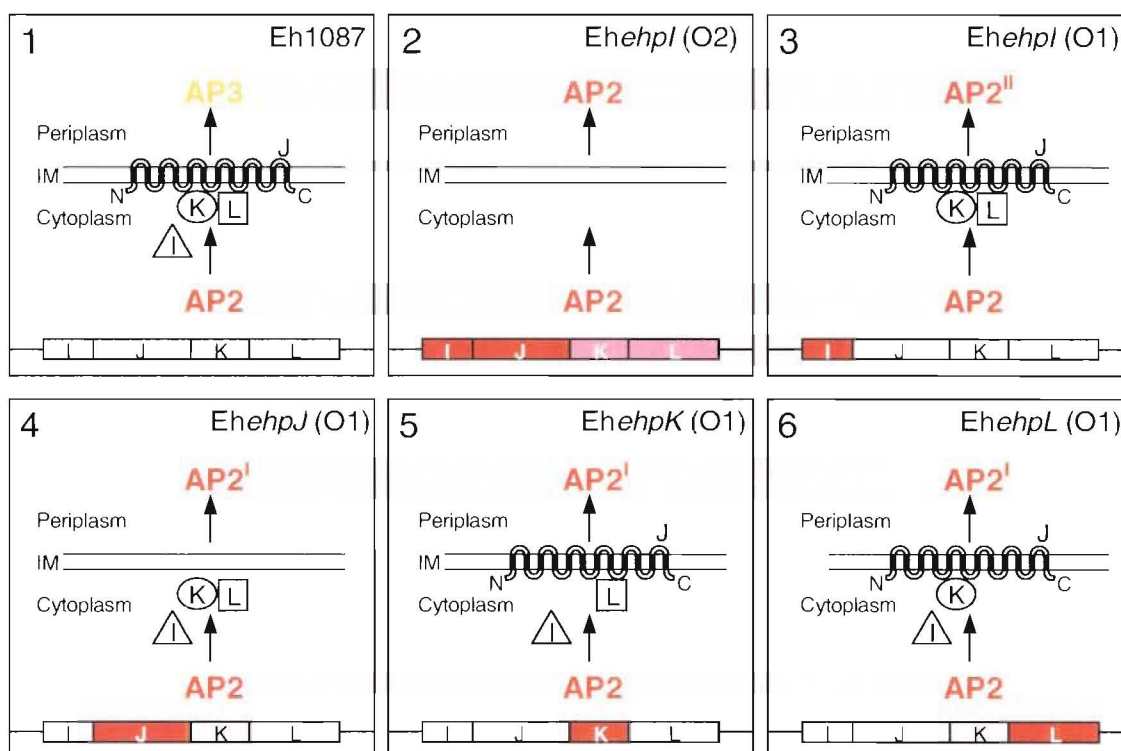


Figure 3.11. Proposed modification and release of AP2 by Group 3 mutants.

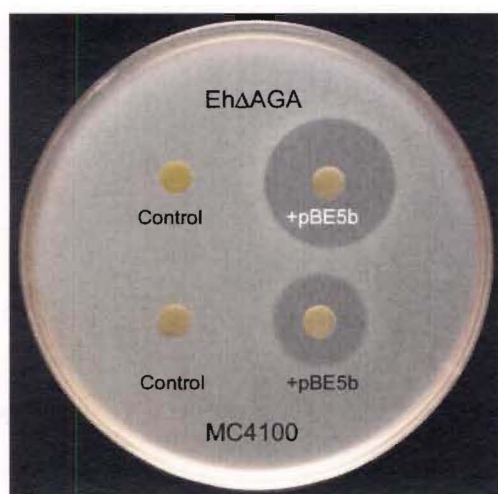
Each panel illustrates the predicted scenario resulting from mutation to a Group 3 gene. The Group 3 gene cluster is drawn at the bottom of each panel, in which inactive genes are highlighted in red. In panel 2 the genes with reduced expression (*ehpK* and *ehpL*) are highlighted in pink, and the translation products of these genes are coloured grey to represent abnormally low concentration. The Group 3 gene products are arbitrarily drawn as polygons, with the exception of EhpJ, which is depicted as a transmembrane protein spanning the inner membrane (IM). AP2, AP2^I, and AP2^{II} are all red compounds released by Group 3 mutants as discussed in the text.

3.6.4. The Group 4 gene products.

The Group 4 gene products are responsible for the conversion of AP3 to AGA as evidenced by the inability of Group 4 mutants to carry out the final conversion of AP3 to AGA (Fig. 3.4) and because the Group 4 genes confer the ability to convert AP3 to AGA on a host cell in the absence of all other *ehp* genes (Fig. 3.12). The products of this group are also capable of catalyzing the addition of a D-alanine residue to GA, the compound created by hydrolysis of

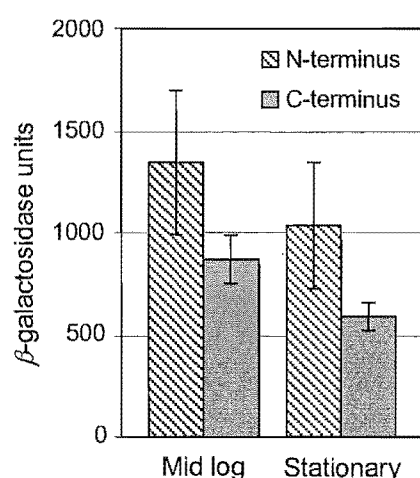
AGA (Figs. 3.4A, 3.12). The predicted protein sequences of both EhpM and EhpN are similar to non-ribosomal peptide synthetases. The sequence of EhpM is most similar to SyrB1, a threonine-binding component of syringomycin synthetase found in *Pseudomonas syringae* pv. *syringae* (Guenzi et al., 1998). PROSITE analysis of EhpM suggests that it belongs to the AMP-dependent synthetase and ligase protein family (residues 42 – 420). In addition, the TMHMM program predicts that EhpM is composed of a cytoplasmic 75 residue N-terminal region, followed by a transmembrane helix of 19 residues, and that the final 397 residue C-terminal region is periplasmic. This topology is supported by the activity of *lacZ* fusions within *ehpM* (Fig. 3.13). Because β -galactosidase is only active in the cytoplasm, the lower activity of a hybrid EhpM::LacZ protein containing 281 amino acids predicted to be periplasmically-localized, compared to a similar fusion with only 80 predicted periplasmic amino acids, suggests that the greater the length of the periplasmic region, the greater the chance that the EhpM protein section can mobilize the fused LacZ protein into the periplasm where it is not active. Alternatively, the β -galactosidase activity of the two EhpM::LacZ proteins may result from structural constraints on LacZ. Additional EhpM::LacZ fusions, particularly to the predicted cytoplasmic region of EhpM, are required to strengthen or discredit this proposal.

Figure 3.12. The Group 4 genes are sufficient for the conversion of AP3 to AGA



Each antibiotic disk contains 20 μ g of AP3 and ca. 10^7 cells of either *E. herbicola* Eh Δ AGA or *E. coli* MC4100 (controls), or these strains harbouring plasmid pBE5b (encodes all Group 4 genes, *ehpM*, *ehpN*, and *ehpO*). Viable cells were recovered from the control disks but not from disks in which AP3 had been converted to AGA. Griseoluteic acid (GA) was similarly converted to AGA by these strains.

Figure 3.13. Support for localization of the C-terminus of EhpM to the periplasm



β -galactosidase activity of *lacZ* fusions to the approximate N-terminal and C-terminal regions of the predicted periplasmic region of EhpM. The N-terminal fusion creates a hybrid protein consisting of LacZ fused to 174 EhpM amino acids, of which the first 75 are cytoplasmic, the next 19 span the inner membrane, and the last 80 are periplasmic. The C-terminal fusion consists of LacZ fused to 375 EhpM amino acids, of which the last 281 are predicted to be periplasmic. Error bars for two standard deviations from three experimental replicates are shown.

3.7. Operon structure and regulation within the *ehp* gene cluster

3.7.1. A system for the creation of Eh1087 *recA* mutants

Complementation studies of Eh1087 (Ant-) mutants and various subclones from the region involved in antibiotic production have produced inconsistencies probably as a result of RecA-mediated homologous recombination between the introduced plasmid DNA and the genome of the host Eh1087 strain (Kearns, 1993). To overcome these problems and accurately assess clones for their ability to complement Eh1087 (Ant-) mutants, a system for marked mutation of the *recA* gene in Eh1087 was developed. Initially, cosmid pL*RecA*, encoding the Eh1087 *recA* gene, was isolated from the Eh1087 genomic library by complementation of DH5 α *recA*. Because the *recA*⁺ allele is normally dominant over most *recA*⁻ mutant alleles, DH5 α *recA*⁺ strains can be differentiated from *recA*⁻ strains by selection for MMS or UV resistance, which requires RecA induction of the SOS response to counter the effect of DNA damage caused by MMS or UV (Owttrim and Coleman, 1987). This selection was used to isolate a 1.8 kbp *Eco*R1 fragment of cosmid DNA containing a functional *recA* gene (pKS*recA*). DNA sequence of this fragment was determined for one strand (Appendix A2.6), which was sufficient to demonstrate very high sequence similarities to the translated *recA* gene products from *Yersinia pestis* (299/331 identities), *Serratia marcescens* (297/330 identities) and *Pantoea agglomerans* (296/331 identities), amongst others. The GeneMark algorithm predicted a gene between an ATG start codon at base 134 and a TGA stop codon at base 1201 (1068 bp) of the 1784 bp

insert DNA in pKS*recA* (Fig. A7). This sequence data, and the complementation of the *recA* mutation in DH5 α , indicated that clone pKS*recA* encodes a 1068 bp *recA* gene from Eh1087.

A *recA*-mutant derivative of pL*recA*, pL*recA*777 was created by inserting an Ω Cm^R cassette between bases 777 and 778 of the Eh1087 *recA* gene to create the mutant *recA* allele *recA*777. The transfer of pL*recA*777 from an *E. coli* S17-1 donor to Eh1087 strains by conjugation was carried out in the presence of Cm to select for maintenance of *recA*777, and in the absence of Tc to ensure that the cosmid vector was not maintained by selection. Under these conditions, 90 – 95 % of all transconjugants were found to be *recA* recombinants that had lost (or never established) the cosmid vector. If the cosmid vector was allowed to establish, recombination occurred at a very low rate and was often difficult to detect. All Eh1087 *recA* strains created by this process were killed by 0.125 mM MMS, or a brief dose of UV irradiation, conditions that differentiate between *recA*⁺ and *recA*⁻ strains (Fig. 3.14). All *recA* strains were confirmed by Southern hybridization (data not shown).

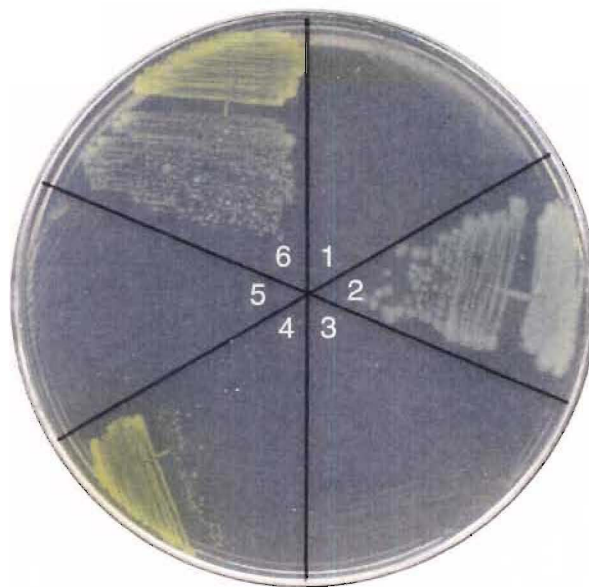


Figure 3.14. MMS sensitivity of Eh1087 *recA* strains

Qualitative assay for RecA activity. The following bacterial strains were streaked on LB agar medium supplemented with 0.125 mM MMS; 1, *E. coli* MC4100 *recA*⁻; 2, MC4100 *recA*⁺; 3, Eh1087 *recA*⁻; 4, Eh1087 *recA*⁺; 5, Eh Δ AGA *recA*⁻; 6, Eh Δ AGA *recA*⁺. Strains 2, 4, and 6 have grown without inhibition by MMS, whereas strains 1, 3, and 5 are all inhibited.

3.7.2. The Eh1087 *recA* mutation is dominant

E. coli MC4100 *recA*⁺ possessing plasmid pKS*recA777* was more sensitive to UV than cells without this clone or than cells possessing pKS*recA*. This indicated that the introduced *recA777* allele was overriding the effect of the functional *recA* gene present in MC4100, and may have been the reason behind the low recombination frequency of Eh1087 strains in which pL*ArecA777* had become established. The chloramphenicol resistance cassette used to create the *recA777* allele contains numerous translation and transcriptional stops at each end. Therefore, it is likely that the 777 bp 5' region of the *recA* gene upstream of the Ω Cm^R cassette may be expressed as a truncated 259 residue protein that can compete with or inhibit the activity of functional RecA monomers.

3.7.3. Recombination rates in Eh1087 *recA*⁺ and *recA*⁻ strains

The creation of *recA* strains was undertaken to prevent homologous recombination occurring between the Eh1087 genome and introduced plasmids. To compare homologous recombination in *recA*⁺ and *recA*⁻ strains of Eh1087, the frequency of recombination between *recA*⁺ and *recA*⁻ strains of mutants *EhehpA* or *EhehpC* and pLA272, cosmid with DNA complementary to the mutation points, was compared. Plasmid DNA prepared from each of these strains was introduced to *E. coli*, and plasmid-containing transformants were isolated on media supplemented with Tc. These transformant were subsequently assessed for the presence of the transposon mini-Tn5*lacZ2* by selection on media supplemented with Km. Tc^R, Km^R co-transformants could only conceivably have arisen from homologous recombination between the Eh1087 mutant genome and the complementary pLA272 DNA. Of 100 Tc^R transformants arising from the introduction of plasmid DNA from one of the four strains, Km^R recombinant plasmids were only isolated from DNA derived from the *recA*⁺ strains, and at a frequency of 16 % for both strains. This frequency was shown to be coincidentally the same, since recombination between pLA272 and other *ehp* mutants ranged from 1.3 % to 17 %, presumably depending on how soon after inoculation a recombination event occurred in each culture.

3.7.4. Complementation of *EhehpA*.

The inability of mutants with O2 transposon insertions to convert exogenous AGA intermediates to AGA suggested that the interruption of any AGA synthesis gene affects expression of all genes transcribed downstream. On this basis it was possible that the 15 AGA-synthesis genes are transcribed solely as a single operon subject to polarity (the single operon hypothesis). An alternative, but not mutually exclusive, explanation is that regulation of gene expression within the AGA-gene cluster could involve *trans*-acting mechanisms. The direction of transcription throughout the 15 *ehp* synthesis genes, and the fact that O2 mutants could not

carry out any functions involving genes downstream of the insertion point, supported the former proposal.

To test the single operon hypothesis, a classical DNA-DNA complementation approach was undertaken, in which a second copy of the *ehp* gene cluster (pLA272) harbouring O2 transposon insertions in one of the *ehp* genes was assessed for ability to transform Eh1087 O2 mutants into antibiotic producers. O2 insertion mutants were used because the transposon orientation ensures that transcription initiated from within the transposon is directed against transcription of the *ehp* genes, creating mutations that demonstrate the influence of an insertion in a particular gene on expression of all downstream genes. In contrast, transcription presumably continues under a Km^R promoter for genes downstream an O1 transposon insertion and polar effects are avoided. Therefore, for all genes downstream of the insertion point in O2 mutants those controlled by the same promoter will not be active, whereas those controlled by a separate promoter will be active. Activity can be detected as antibiotic production if a second copy of the *ehp* gene cluster harbouring a transposon insertion in another *ehp* gene is provided in trans (Fig. 3.15). For this investigation, a selection of O2 transposon insertions in each *ehp* gene, except *ehpE* and *ehpO*, were transferred to cosmid pLA272 by homologous recombination. Each cosmid derivative created by this process was initially introduced to an *EhehpA* O2 mutant that had been modified to be *recA* to prevent homologous recombination between the introduced DNA and the mutant genome. It was anticipated that if the single operon hypothesis was correct then no cosmid derivative should complement *EhehpA* (O2) because no strain would have all the gene products required for AGA synthesis. In contrast, pLA272 with O2 insertions in *ehpF-ehpO* did complement *EhehpA* indicating that these genes are expressed independently of the Group 1 genes (Fig 3.15).

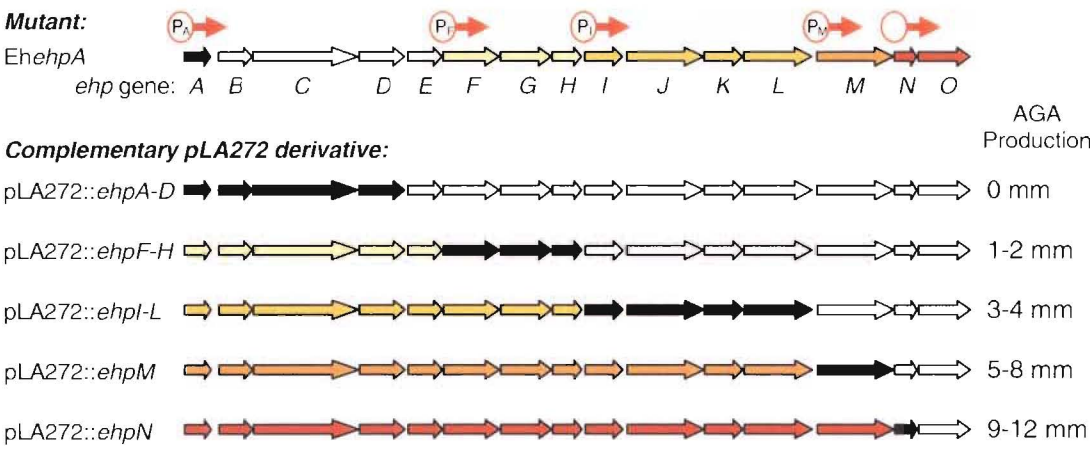


Figure 3.15. *EhpA* complementation by *pLA272* transposon-insertion derivatives

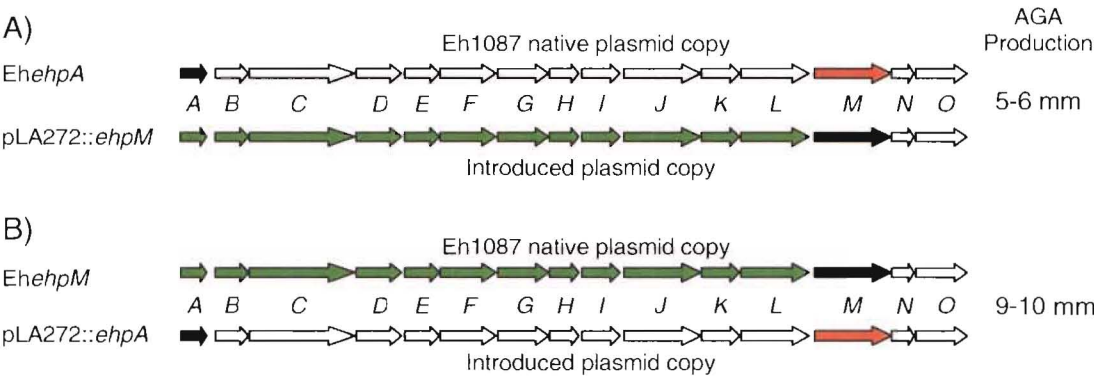
Black arrows represent genes interrupted by mini-Tn5lacZ2, and white arrows represent genes for which expression is unknown. Gene activity (either gene expression or functionality of the gene product) is colour coded; yellow arrows represent low activity, through to red arrows, which represent high activity. Antibiotic production was measured as 2 x radius of halo from edge of colony, and in all cases results from *trans* complementation of the genomic *ehpA* mutation. Five predicted promoters, P_A , P_F , P_I , P_M , and P_N , are shown at the top of the diagram.

Strains of *EhpA* harboring a second copy of the AGA-gene cluster mutated in a Group I gene (*pLA272::ehpA*, *B*, *C*, or *D*) do not produce antibiotic. Since all of these strains are lacking at least one Group I gene product, the genes *ehpA-ehpD* must be expressed as an operon from a single promoter. Plasmids *pLA272::ehpF*, *pLA272::ehpG*, or *pLA272::ehpH* partially complemented *EhpA*, resulting in low level antibiotic production. Therefore, a second promoter controls expression of *ehpF*, *ehpG*, and *ehpH* as an operon, and expression from this promoter is either secondary to read-through from the *ehpA* promoter, or is reduced when the preceding genes are interrupted. Further analysis of the *EhpA* complementation data indicated that the expression of all genes tested, except *ehpN*, is reduced when any upstream *ehp* gene is interrupted. the OI mutations re-initiate This observation suggests that most genes required for AGA synthesis have an underlying operon structure, with additional promoters controlling each functional gene group over and above, or in the absence of, the underlying transcription of the complete ‘AGA synthesis operon’.

Table 3.4. Complementation of Eh1087 O2 mutants

	pLA	pLA272 derivatives ¹													controls ²	
		A	B	C	D	F	G	H	I	J	K	L	M	N	1µg	Eh
EhehpA	11	0	0	0	0	1	2	2	3	3	3	3	6	11	4	16
EhehpF	12	2	3	3	2	0	0	0	1	1	1	1	5	11	5	17
EhehpJ	12	6	6	6	5	2	2	2	2	0	1	1	5	11	5	16
EhehpM	12	9	9	10	9	7	7	7	7	7	3	3	0	7	4	16

Antibiotic production by *recA* mutants of *EhehpA*, *EhehpF*, *EhehpJ* and *EhehpM* harbouring pLA272 derivatives (measured, in mm, as diameter of clearing zone minus colony diameter). Data in *italics* is artificially high due to the effect of plasmid copy number as illustrated in Figure 3.16. ¹pLA272 (pLA) and recombinant derivatives of pLA272 containing a transposon insertion in the *ehp* gene listed (note: excluding *ehpE* and *ehpO*). ²Controls to assess variation between plates were 1 µg AGA (in a six mm Φ disk) and Eh1087 cells cultured and applied as per test strains.



Complementation of an *Ehehp* mutant by a plasmid with a second copy of the *ehp* gene cluster containing a different *ehp* mutation. The genomic gene cluster copy is shown above the plasmid copy. Coloured arrows represent genes with the following properties; green, genes that are subject to normal expression control mechanisms; black, genes harbouring a mutation; white, genes downstream of a mutated gene and subject to expression independent of *ehpA*; red, the copy of *ehpM* that provides a measure of the *ehpM* expression that is independent of *cis* influences from the expression of *ehpA*. A) Antibiotic production by a strain containing both a genomic mutation in *ehpA* and an *ehpM* mutation in a plasmid copy of the gene cluster provides a measure of *ehpA*-independent expression of the genomic *ehpM*. B) *ehpA*-independent (*cis*) expression of the plasmid-encoded *ehpM*.

3.7.5. Gene cluster copy number limits complementation analysis

The copy number of the introduced plasmid containing the AGA gene cluster has an important influence on the interpretation of complementation data. As an example, the complementation of *EhehpA* by pLA272::*ehpM* (A), and *EhehpM* by pLA272::*ehpA* (B) is compared in Figure 3.16. In both situations the Group 1, 2, and 3 genes (*ehpA-ehpL*) are expressed under control of the regular *ehp* gene cluster promoters on either the introduced plasmid (A) or the Eh1087 genome (B), whereas *ehpM* is provided in *trans* by expression from a promoter independent (mostly) of these influences (red arrows). The AGA gene cluster is encoded on a ca. 200 kbp plasmid of unknown copy number in Eh1087. However, since the provision of *ehpM* in *trans* is the limiting factor for antibiotic production by these strains, and antibiotic production is lower when the functional *ehpM* is encoded by the Eh1087 genome rather than the plasmid pLA272::*ehpA*, it appears that the copy number of pLA272 is higher than the native Eh1087 plasmid. In strain *EhehpM* harboring pLA272::*ehpA*, the introduced plasmid presumably supplies artificially high amounts of *EhpM*, and disguises the true effect of an *ehpA* insertion on expression of *ehpM*. For this reason the activity of the *ehp* genes present on the native Eh1087 plasmid was scored to assess complementation, rather than the activity of the *ehp* genes on the introduced plasmid. Unfortunately this approach is limited to assessing the influence of a particular gene on the expression of downstream genes, and does not differentiate between *cis*- and *trans*-acting regulatory mechanisms.

3.7.6. Complementation of Group 2, 3, and 4 mutants.

Since the Group 1, Group 2, and Group 3 genes appeared to be controlled as individual operons, complementation of a representative mutant from each group by pLA272 derivative-plasmids was used as an approximate measure of the influence that each group had on the expression of downstream genes. The plasmid pLA272, and transposon insertion derivatives of pLA272, were introduced to *recA* mutants of *EhehpF* (Group 2), *EhehpJ* (Group 3), and *EhehpM* (Group 4), and all strains were assessed for antibiotic production (Table 3.4). For unknown reasons the amount of antibiotic produced by mutants harbouring pLA272 was markedly less than Eh1087, however because pLA272 complemented all mutants equally it is likely that this difference would have a consistent influence on the complementation analyses reported here. The data presented in Table 3.4 again indicates that the functional Groups 1, 2, and 3, are expressed as individual operons with dedicated promoters, and that expression or activity of most genes is influenced, in varying degrees, by the expression of all genes encoded upstream. This influence provides further clues about the relative strength of the regulatory mechanisms controlling the AGA synthesis gene cluster. For instance, the lower the amount of antibiotic produced by a strain containing two copies of the AGA gene cluster, each with an insertion in different genes,

the stronger the co-regulation of the two mutated genes (as an operon or by other mechanisms), or the greater the likelihood that those gene products act together (Fig. 3.17).

It is proposed that the Group 3 and Group 4 operons are not only preceded by promoters, but also contain the 'intra-operon' promoters, P_K , and P_N , respectively (Figs. 3.15, 3.17). Promoter P_K is present within the Group 3 operon as evidenced by *EhehpJ* harbouring pLA272::*ehpK*. This strain demonstrates that P_K can drive the expression of *ehpK* and *ehpL* at a low level when transcription through *ehpJ* is interrupted. P_K also appears to influence the expression of *ehpM* since antibiotic production by *EhehpM* harbouring pLA272::*ehpK* / *L* is far lower than other *EhehpM* strains. If the expression of *ehpM* was not linked to *ehpK*, surplus *ehpM* would be provided *in trans* (due to the higher plasmid copy number), and this strain would produce a similar amount of antibiotic as *EhehpM* harbouring pLA272. The Group 4 genes *ehpM* and *ehpN* have separate promoters as demonstrated by antibiotic production by *EhehpM* harbouring pLA272::*ehpN*, and by the increased production of all strains harbouring pLA272::*ehpN* compared to strains harbouring pLA272::*ehpM*. The relatively large amount of antibiotic produced by this strain indicates that the *ehpN* promoter is reasonably active in the absence of *ehpM*, in contrast to the low activity of the *ehpK* promoter in the absence of *ehpJ*. However, because mutants *EhehpA*-*EhehpL* harbouring pLA272::*ehpN* produce more antibiotic than *EhehpM* (pLA272::*ehpN*), full expression of *ehpN* must require a functional *ehpM* gene. Therefore, the Group 4 genes probably have an underlying operon structure, much like the other functional groups, and the expression of *ehpN* is also controlled by a secondary 'intra-operon' promoter (P_N).

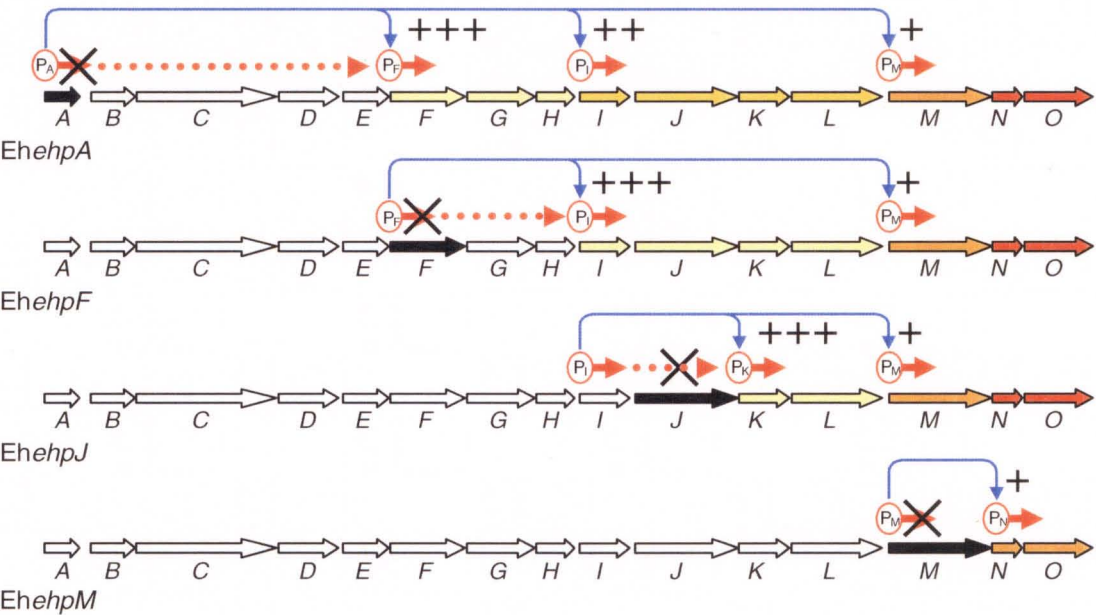


Figure 3.17. The polar effect of *ehp* mutations on the expression of downstream genes.

Gene activity is colour coded as per Fig. 3.15. Dotted red lines represent operons, and the black crosses highlight the point at which transcription is interrupted by an O₂ transposon insertion in the gene coloured black. The influence of one gene on the ‘activity’ of another (blue arrows) was graded as follows:

- +++ strong dependence of one gene on another; for example *EehpA* is only weakly complemented by a second copy of the gene cluster with an insertion in *ehpF*, *ehpG*, or *ehpH*. Strains produce a 1-2 mm Φ antibiotic halo (yellow arrows).
 - ++ moderate dependence, strains produce a 3 mm Φ antibiotic halo (light orange arrows) .
 - + weak dependence, strains produce a 5-7 mm Φ antibiotic halo (dark orange arrows).
- (red arrows depict genes that are not influenced by a mutation in an upstream gene)

3.7.7. An assessment of post-transcriptional regulation

Complementation studies indicated that there are six promoters driving expression of the *ehp* genes, and that if transcription from P_A is interrupted, the activity of all promoters downstream of Group 1 is very low (Figs. 3.15 - 3.17). Therefore, the *ehp* genes appear to be expressed predominantly as a single operon (excluding *ehpN* and presumably *ehpO*) under control of P_A . If *ehp* gene expression is regulated solely at the level of transcription, then expression of all genes should be similar or greater for genes further from *ehpA* as additional promoters could potentially enhance the transcription of such genes. To quantify the expression of the *ehp* gene translation products, and determine whether the expression of these products is subject to regulation at the level of translation, the activity of in-frame, *lacZ* fusions to these genes was measured. This approach enabled the expression of the translation products of *ehp* genes from different groups (*inter-group*), and from within the same group (*intra-group*), to be compared

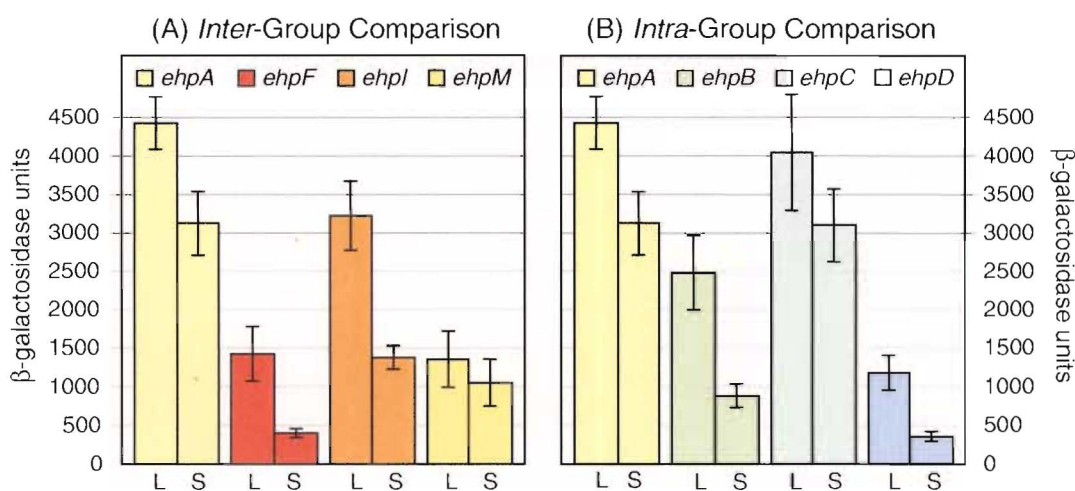


Figure 3.18. β -galactosidase activity of *Eehp* mutants with active *lacZ* fusions.

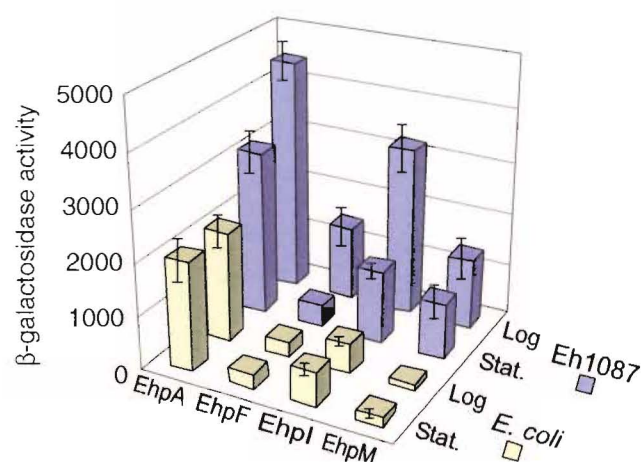
Mutants were grown in 1-A minimal media to an optical density ($\lambda = 600$ nm) of 0.4-0.6 (mid-log phase, L), or for 24 hours (stationary phase, S), and β -galactosidase activity was measured by the method of Miller.

The expression of representative genes from Groups 2, 3, and 4, were all lower than *EhpA*, indicating that the *ehp* gene cluster is subject to transcriptional and translational regulation, and

that the activity of the secondary promoters upstream of each of these groups is insufficient to override the translational regulation (Fig. 3.18A). Furthermore, the regulatory mechanism(s) controlling the rate of translation are highly influential even within the Group 1 genes (Fig. 3.18B). There are also examples of intra- and inter-group variation in the suppression of gene expression during entry to stationary phase. The production of most gene products decreases in varying degrees during this period, except EhpC and EhpM, indicating that there is a second post-transcriptional regulatory mechanism. Thus, translation of the AGA-gene cluster transcript is differentially regulated for each gene during exponential-phase growth, and the magnitude of the suppression of translation during entry to stationary phase is also differentially regulated for each gene tested in this study.

To determine whether or not these translation-regulating mechanisms are specific to Eh1087 and could affect the amount of AGA produced by Eh1087 relative to *E. coli*, the expression of LacZ::Ehp fusion proteins was measured in both bacteria. To assess the expression of these fusions in *E. coli*, the appropriate mini-Tn5lacZ2 insertion derivatives of pLA272 were introduced to *E. coli* MC4100, a strain that produces AGA when it contains pLA272, although with far lower efficiency than Eh1087. The copy number of pLA272 is likely to be higher than the copy number of the native Eh1087 plasmid, and this may result in an over-estimation of *ehp* gene product expression in *E. coli* relative to Eh1087. As shown in Figure 3.19, even with a copy number effect, the expression of the gene fusions is far lower in *E. coli* MC4100 than in Eh1087. For instance, the expression of EhpA during log phase is ca. 50 % less in MC4100 than in Eh1087, and the expression of EhpF, EhpI and EhpM is 80-90 % less in MC4100.

Figure 3.19. Relative expression of Ehp proteins in Eh1087 and *E. coli*.



Comparison of EhpA, EhpF, EhpI and EhpM expression in log and stationary phase (Stat.) cultures of Eh1087 and *E. coli* MC4100. Expression is measured as β-galactosidase activity resulting from the expression of LacZ fused to each Ehp protein. Error bars show two standard deviations (from 3 replicates) where the deviation is greater than 100 β-galactosidase units.

3.8. The AGA-gene cluster contains a resistance gene

3.8.1. Localization of the AGA-resistance gene

Three cosmids, pLA272, pLA203, and pLA214, conferred AGA-resistance on an *E. coli* host cell as shown in Figure 3.1. The plasmid pBB7, containing a 7 kbp DNA fragment from pLA272 (Fig. 3.2) also provided *E. coli* with AGA resistance. To localize the resistance gene(s) random insertions of mini-Tn10*lacZ'* in pBB7 were isolated and screened for loss of AGA-resistance in an *E. coli* host. Such insertions were mapped by restriction enzyme fragment analysis to within a 400 bp region upstream of *ehpA* (Fig. 1B). Active *lacZ* fusions that resulted in a resistance-minus phenotype were orientated so that *lacZ* was divergently transcribed from the rest of the *ehp* genes (Fig. 3.5). Analysis of the DNA sequence surrounding these transposon insertions using GeneMark indicated that they interrupted a 390 bp gene, designated *ehpR* (AGA resistance), which possessed no similarity to any DNA or protein sequences in the databases (December, 2001). The translation product of *ehpR* was predicted to be a protein of 14.6 kDa, which agrees with the size of the EhpR protein expressed in *E. coli* minicells (Fig. 3.6).

3.8.2. *ehpR* is necessary and sufficient for AGA-resistance in Eh1087 and in *E. coli*

Three lines of evidence suggest that immunity is necessary for Eh1087 survival during antibiotic production. Firstly, no insertions to the resistance gene were isolated during the screening of 5000 Eh1087 mini-Tn5*lacZ2* mutants. Secondly, although transposon insertions either side of *ehpR* could be transferred from pBB7 to the Eh1087 genome by recombination at a frequency of 1-2 % no recombination was detected for equivalent insertions within the resistance gene (data not shown). Thirdly, and perhaps most convincingly, Eh Δ AGA cells containing the Group 4 genes on a plasmid can convert exogenous AP3 to AGA, but die in the process due to sensitivity to AGA (Fig. 3.12). Based on the assumption that the resistance factor is essential for Eh1087 survival, and therefore that *ehpR* could only be mutated if antibiotic synthesis was first inactivated, both the AGA-resistance and biosynthesis regions were deleted from E*ehpA* to create Eh Δ AGA. All four resulting Eh Δ AGA strains were sensitive to AGA (minimal lethal concentration (MLC), 1.6 $\mu\text{g}.\text{ml}^{-1}$), and were transformed to be antibiotic resistant by pBB7 or pBB7::*ehpA* (MLC >51.2 $\mu\text{g}.\text{ml}^{-1}$), but not pBB7::*ehpR*. Similar results were obtained with *E. coli* strains harbouring these plasmids, demonstrating that the resistance factor is sufficient for antibiotic resistance in Eh1087 and *E. coli*. Furthermore, the provision of resistance to *E. coli* demonstrates that EhpR acts independently of the AGA-synthesis gene cluster and any other Eh1087-specific factors.

3.8.3. EhpR may prevent AGA from entering an otherwise susceptible cell

The predicted amino acid sequence of EhpR does not have features found in membrane-localized proteins such as transmembrane α -helices or hydrophobic regions, thus it is unlikely to associate with a cell membrane and function as a drug efflux pump. As discussed in Chapter 4, AGA has redox potential and could be oxidized to a semiquinone free radical (see Fig. 4.11), a compound that would have the potential to react with many cell components and cause wide ranging cellular damage. If this is the case then it is unlikely that EhpR protects or alters a specific AGA target. To test whether EhpR acts to inactivate AGA or prevent AGA uptake, AGA was exposed to Ea8862 and Ea8862 (*ehpR*), re-extracted after various exposure periods, and assessed for activity (Fig. 3.20). After 60 min., all Ea8862 cells exposed to excess AGA (25 times MLC) were killed, and all AGA activity was lost, demonstrating that AGA loses activity in the presence of Ea8862. This result suggests that AGA reacts with some cell component(s), losing activity in the process. Furthermore, AGA appears to react with and become inactivated by even dead cells, because AGA inactivation continued when all Ea8862 cells had died (For example, see Fig. 4.7). Live cells, for instance Ea8862 (pEhpR), appear to degrade AGA at a slightly faster rate than dead cells suggesting that recently killed cells, in which metabolism is reduced but still potentially active, are less reactive to AGA. In contrast, the reactivity of a bacterium with intrinsic resistance to AGA, *Pseudomonas aureofaciens* PA147-2, was found to be considerably less than either Ea8862 or Ea8862 (pEhpR).

3.8.4. The AGA synthesis and resistance genes are not necessary for plasmid maintenance *in planta*

The AGA gene cluster contains genes for AGA synthesis and resistance, thus it was possible that the gene cluster could act as a plasmid maintenance system whereby all cells that segregated without the plasmid would also lack AGA resistance and may be killed by neighbouring AGA-producing cells. It was hypothesized that interruption of the AGA-gene cluster would result in loss of the native plasmid over time. This hypothesis was tested by assessing the loss of Sm^{R} in strain Eh Δ AGA, which was created by replacing the AGA gene cluster with an Sm^{R} marker. To enhance the selection pressure for cells that had lost the metabolic burden of the native plasmid, plasmid loss was assessed in cells of Eh Δ AGA that had been competing with Ea8862 for the stigma of an apple blossom (see chapter 2 for experimental details). Strains isolated from blossoms after 24, 48, and 72 h were enumerated on selective media, and each Rf^{R} Eh Δ AGA colony was assessed for loss of the plasmid marker, Sm^{R} . It was assumed that the establishment of Ea8862 on stigmas before inoculation with Eh Δ AGA would provide a difficult environment for Eh Δ AGA to grow, and therefore increase the selection pressure for loss of excess metabolic burdens, such as a plasmid, even further. However, no Rf^{R} strains isolated from blossoms after 24 h (95 colonies), 48 h (120 colonies), or 72 h (106

colonies) were Sm^{S} , indicating that the Eh1087 plasmid is maintained by some mechanism other than the provision of AGA synthesis and resistance to a host cell.

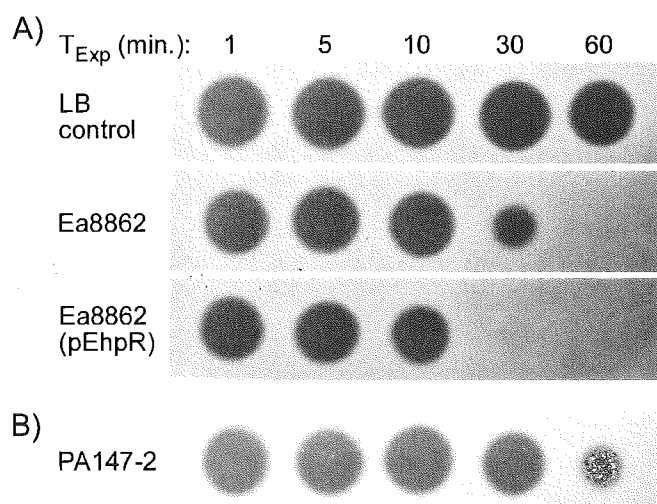


Figure 3.20. The influence of EhpR on AGA degradation by bacterial cells

LB media supplemented with $10 \mu\text{g}.\text{ml}^{-1}$ AGA and either no bacteria (LB control), Ea8862, Ea8862 (pEhpR), or PA147-2 were incubated at 30°C and AGA was extracted to chloroform from $200 \mu\text{l}$ ($0.2 \mu\text{g}$ AGA) of culture supernatant at the designated times. Over the 60 min period of the assay the Ea8862 population decreased from 1.2×10^9 to $< 10 \text{ cfu}.\text{ml}^{-1}$, whereas the Ea8862 (pEhpR) population did not decrease ($6.5 - 5.8 \times 10^8 \text{ cfu}.\text{ml}^{-1}$) and the PA147-2 population decreased by an intermediate amount (2.2×10^9 to $5.2 \times 10^7 \text{ cfu}.\text{ml}^{-1}$). MIC data for these strains: Ea8862, $0.25 \mu\text{g}.\text{ml}^{-1}$; Ea8862 (pEhpR), $>51.2 \mu\text{g}.\text{ml}^{-1}$; PA1472 $>16 \mu\text{g}.\text{ml}^{-1}$ (see section 4.5).

3.9. Origin of the AGA gene cluster.

3.9.1. Amongst *E. herbicola* the AGA gene cluster may be unique to Eh1087

Twelve *E. herbicola* strains isolated from various field locations in New Zealand were investigated for the presence of all or part of the DNA region responsible for antibiotic production (strains were obtained from the International Collection of Micro-organisms from Plants, Landcare Research / Manaaki Whenua New Zealand Ltd., Auckland, New Zealand). Genomic DNA from these strains failed to hybridize to the DNA of cosmid 3272, or subclones pBB7, pBE5a, and pBE5b which cover the AGA-gene cluster region indicating that neither the AGA gene cluster, nor part thereof, is present in the *Erwinia* spp. investigated (data not shown). Since it has been shown that the AGA-gene cluster is located on a large plasmid (Kearns, 1993; M. Galbraith, pers. comm., 2001) there is a good chance that the entire plasmid may be unique to Eh1087.

3.9.2 DNA sequence characteristics suggest that the AGA gene cluster did not originate in *Pseudomonas* spp.

The lack of similarity between the DNA sequences of the AGA gene cluster and the described PCA operons is at least partly due to the considerable difference in GC content between them, as illustrated in Figure 3.21. The AGA gene cluster has a GC content of 43.7%, whilst the PCA operons vary from 61.2-68.8 % (accession numbers: *Pseudomonas aeruginosa*, AF005404; *P. aureofaciens* 30-84, L48339; *P. chlororaphis* PCL1391, AF195615; *P. fluorescens*, L48616).

3.9.3 Evidence that the AGA-gene cluster is a transposon

Analysis of the DNA sequence of the regions upstream and downstream of the AGA gene cluster suggests that the cluster may be part of a transposon. In both regions there are similarities of varying significance to transposase-type genes (see Appendices A2.2 and A2.3). The probable transposase encoded in the region upstream of *ehpR* has a far higher GC content than the remainder of the gene cluster, suggesting that it is of different origin (Fig. 3.21). The DNA sequence of a possible transposase-encoding region downstream of *ehpO* is short (ca. 1000 bp) but also appears to mark a transition to higher GC content. The sequence of DNA beyond this region is required to strengthen or refute this proposal.

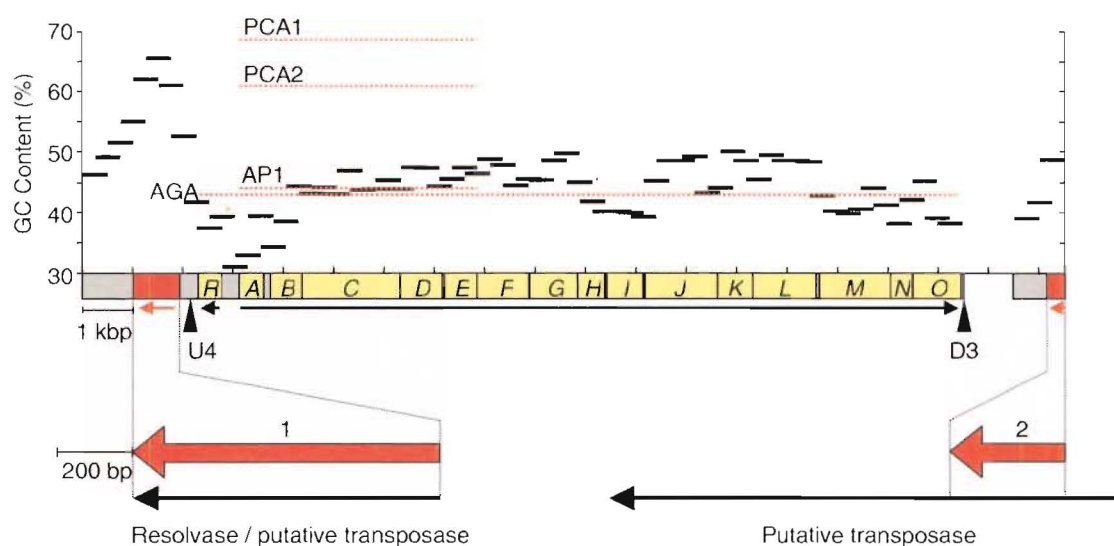


Figure 3.21. DNA sequence properties of the AGA gene cluster and neighbouring DNA.

The DNA sequence of the region illustrated in grey has been determined, including the *ehp* genes (yellow). The boundary-defining insertions U4 and D3 are shown for comparison with Figure 3.2, and the direction of transcription within the DNA region is shown by arrows below the gene cluster. The variation in GC content throughout the sequenced region is shown in the top section, in which short black lines represent the GC content of overlapping 500 bp sections of DNA sequence. The average % GC content of the *ehp* gene cluster is shown by a dashed red line (AGA). The dotted red lines indicate the average GC content of the phenazine nucleus genes in *Ps. aeruginosa* PAO1 (PCA1), in *Ps. fluorescens* 2-79, *Ps. chlororaphis* PCL1391, and *Ps. aureofaciens* 30-84 (PCA2), and in *E. herbicola* Eh1087 (AP1). Regions with sequence similarity to the translated protein products of plasmid-based transposase / resolvase genes are illustrated in the lower section of the diagram (see Appendix 2). Arrow 1 represents similarity to the complete sequence of a probable transposase from *Yersinia enterocolitica* (8e⁻⁵⁹), and to the F-plasmid resolvase gene (2e⁻¹¹), whereas Arrow 2 illustrates similarity to part of the sequence found in putative transposases from various sources (up to 1e⁻¹⁰).

Discussion

The characterization of antibiotic gene clusters provides information that is useful for the manipulation of antibiotic biosynthesis pathways to alter the ability of an organism to produce different amounts of antibiotic in different environments, or even to produce antibiotic variants. The genetic modification of an antibiotic gene cluster in such a way offers enormous potential for the development of superior biological control agents for which antibiosis is important for pathogen suppression. Given that antibiosis is recognized as important for suppression of the Fire Blight pathogen, *E. amylovora*, by many strains of *E. herbicola*, it is somewhat surprising that there is so little data about the molecular genetics of antibiotic production in these strains. In this regard, the present study breaks new ground in characterizing a substantial 16-gene cluster required for synthesis of AGA by Eh1087, an antibiotic that is involved in the suppression of *E. amylovora* (see chapter 2; also Kearns, 1993; Kearns and Mahanty, 1998). Just as importantly, this is the first description of genes required for synthesis of the phenazine nucleus outside of the *Pseudomonas* genus. The results presented in this chapter also demonstrate the general roles of sub-clusters of genes in the modification of the phenazine nucleus (PCA); in contrast, the modification of PCA in *Pseudomonas* spp. appears to involve single genes, each involved in a particular PCA modification (Chin-A-Woeng et al., 2001; Delaney et al., 2001; Mavrodi et al., 2001). Also of significance is the finding of a dedicated phenazine antibiotic resistance gene associated with the AGA-gene cluster in Eh1087, since to the authors knowledge no such gene has been described previously.

Previously, transposon mutants of Eh1087 were isolated that could not produce antibiotic (Kearns, 1993). Characterization of the transposon insertion points demonstrated that a region of DNA required for antibiotic production in Eh1087 was located on a plasmid, and concluded with the cloning and sequencing of four putative genes within the region that were essential for antibiotic production (Kearns, 1993; Kearns and Mahanty, 1998). These data and tools provided a good starting point for the characterization carried out in the present study. The limited number and distribution of transposon insertions isolated in the previous investigation indicated that the original mutagenesis screen not only underestimated the AGA-gene cluster size, but may also have missed additional genetic elements required for antibiotic production in Eh1087. In the present investigation, the use of defined insertion points, cloning, and a second mutagenesis screen has now demonstrated the extent of a gene cluster sufficient for antibiotic production, and revealed two non-gene cluster loci that may be involved in this process.

Characterization of the AGA-gene cluster genes

Characterization of the AGA gene cluster is the predominant theme of this thesis. A primary goal was the elucidation of the genes and their respective role(s) in the production of AGA. Gene predictions were based on DNA sequence data, and supported by empirical evidence in the form of expressed gene protein products. Preliminary investigations into the function of each gene were helped enormously by the production of coloured AGA intermediates by various Eh1087 AGA-minus mutants, a feature also noted for phenazine mutants in other bacteria (Byng et al., 1979; Byng and Turner, 1976; Carson and Jensen, 1974). This observation led to the use of classical syntrophism tests, as outlined by Hayes (1968), in which the ability of mutants to 'cross feed' each other and to convert antibiotic intermediates into AGA demonstrated the role of each gene (product) in the process of AGA synthesis (Figs. 3.4, 3.7). Complementation analysis provided information about the operon structures within the gene cluster, and thus the probable site of promoters for the initiation of transcripts, and the comparison of this data with the expression of active *lacZ* fusions to various *ehp* genes gave clues about the transcriptional and translational expression of the AGA-biosynthesis genes. In summary, the AGA gene cluster in Eh1087 has been comprehensively described, and preliminary information about the function and expression of each gene has been obtained.

Several studies have established that a seven-gene operon is necessary for the synthesis of the phenazine nucleus (PCA) by fluorescent *Pseudomonas* spp., and predicted or demonstrated authentic roles for each gene in the process (Fig. 3.8). In contrast, the AGA gene cluster of Eh1087 is composed of 16 *ehp* genes, of which five genes have similar products to the PCA operon. The AGA gene cluster contains an additional eleven genes required for modification and export of the phenazine nucleus, and a final gene whose product provides resistance against the antibiotic activity of AGA. The analysis of individual *ehp* mutants for the production of AGA intermediates, and for the ability to convert intermediates to AGA, enabled the genes responsible for AGA synthesis to be divided into four functional groups (Groups 1-4).

The Group 1 genes (*ehpA-ehpE*) are probable analogs of five of the seven genes required for synthesis of PCA by *Pseudomonas* spp. (Fig. 3.8). In Eh1087 these genes are required for the synthesis of the first coloured AGA precursor, AP1, which has an absorbance spectrum similar to PCA (see Fig. 4.7). The low yield of AP1 and the poor conversion of AP1 to AGA by Group 2 mutants, suggest that AP1 is synthesized within the cytoplasm. Because AP1 and PCA have very similar absorbance spectra and only slightly different solubility in water, it is likely that the chemical structures of these compounds are very similar. The difference between PCA and AP1 could be due to the notable absence in the AGA gene cluster of an apparent gene duplication

responsible for the nearly identical *phzA* and *phzB* (or *phzX* and *phzY*) genes encoded in the PCA operons (Fig. 3.8). PhzA and PhzB influence the relative amounts and types of phenazines produced by *P. aureofaciens* 30-84 and *P. fluorescens* 2-79 (Mavrodi et al., 1998; McDonald et al., 2001), therefore the single analog (*ehpA*) present in Eh1087 may specify the synthesis of AP1 rather than PCA as the first AGA precursor. The AGA Group 1 genes also lack *phzC* (*phzF*), whose product is similar to plant 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP) synthases. DAHP synthases carry out the first step of the shikimic acid pathway and are regulated by numerous mechanisms, for instance bacterial DAHP synthases are often subject to feedback inhibition by aromatic amino acids (Jensen et al., 1967) whereas plant variants of this enzyme are apparently not (Mavrodi et al., 1998). PhzC is more similar to plant DAHP synthases, and as such could maintain shikimic acid synthesis and ensure a constant supply of the phenazine precursor, chorismic acid, in the presence of aromatic amino acids (Mavrodi et al., 1998). The partial suppression of AGA production in Eh1087 by exogenous tyrosine (data not shown) supports the prediction that Eh1087 lacks a functional equivalent of PhzC (PhzF) and cannot override feedback inhibition of the native DAHP. This difference between the PCA operon and the Group 1 genes may affect the relative amount of the phenazine nucleus synthesized by Eh1087 compared to *Pseudomonas* spp. in some environments.

Genes comprising Groups 2, 3, and 4 of the AGA gene cluster encode products responsible for the conversion of the phenazine nucleus (AP1) to AGA by a process that involves at least two coloured intermediates (Fig. 3.23). The equivalent region immediately downstream of the PCA operon in some *Pseudomonas* spp. encodes *phzS*, *phzO*, or *phzH* for the conversion of PCA to 1-OH-PHZ, 2-OH-PCA, or PCN, respectively (Chin-A-Woeng et al., 2001; Delaney et al., 2001; Mavrodi et al., 2001). Since AGA differs from PCN by a carboxamide group, and from 1-OH-PHZ and 2-OH-PCA by a hydroxyl group attached to the phenazine nucleus, it is not surprising that the Eh1087 AGA gene cluster does not appear to possess analogs of *phzH*, *phzO* or *phzS*. It is proposed that the genes in Groups 2, 3, and 4 are responsible for novel phenazine modifications. The only other organism currently known to produce AGA is *Vibrio* strain SANK 73794 which presumably has a similar biochemical pathway for modification of the phenazine nucleus (Sato et al., 1995). However, neither this pathway nor the genetic basis for this pathway in strain SANK 73794 have been investigated as far as the author is aware.

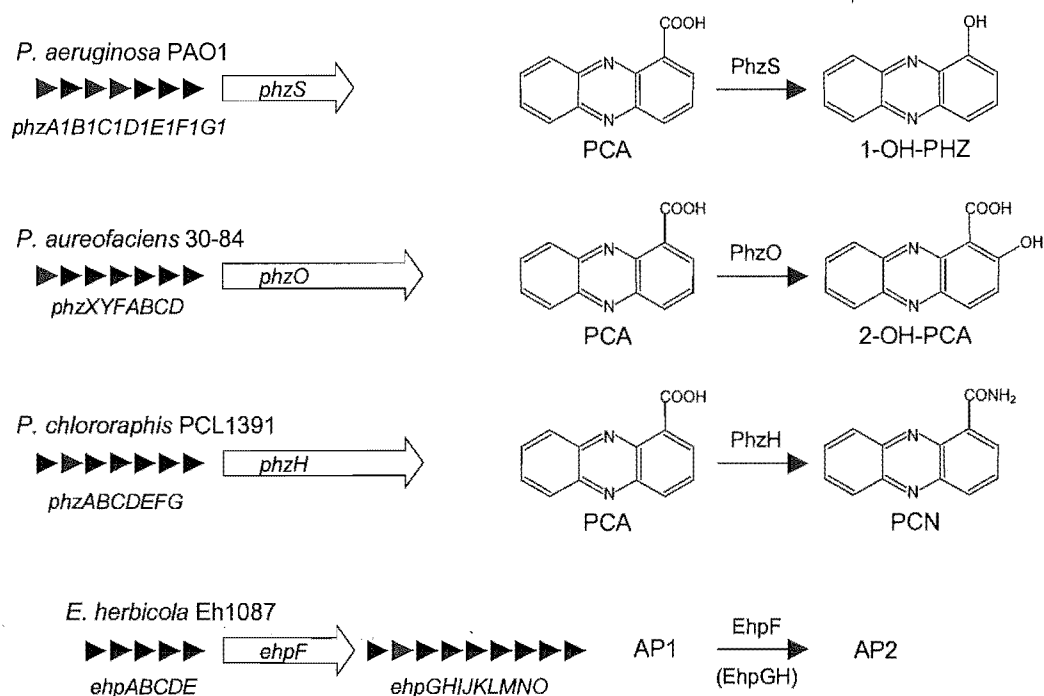


Figure 3.22. Comparison of the genes immediately downstream of the PCA operon

Comparison of genes immediately downstream of the phenazine nucleus operon in *Pseudomonas* spp. and Eh1087. The proposed modification carried out by the products of these genes are shown on the right (adapted from Delaney et al., 2001; Mavrodi et al., 2001). EhpF acts in association with EhpG and EhpH for the conversion of AP1 to AP2. The product of *phzS* (*P. aeruginosa* PAO1) also acts to convert PCA to pyocyanin in association with the product of *phzM*. PCA, phenazine-1-carboxylic acid; 1-OH-PHZ, 1-hydroxy-phenazine; 2-OH-PCA, 2-hydroxy-phenazine-1-carboxylic acid; PCN, phenazine carboxamide.

The three Group 2 gene products, EhpF, EhpG, and EhpH, carry out the conversion of AP1 to AP2. The first member of this group, EhpF, has an AMP-binding domain motif found in proteins that catalyze reactions requiring ATP. Although the direct involvement of ATP in the synthesis of AGA has not been clearly demonstrated, EhpF may act as a sensor of cellular energy levels and limit AGA synthesis during stationary phase when ATP becomes less available, and when antibiotic production by Eh1087 stops (see Figure 4.1). The lack of active *phoA* fusions to *ehpG* and *ehpH* indicates that their translation products are predominantly cytoplasmic. Furthermore, the predicted protein sequences of EhpG and EhpH possess short hydrophobic regions suggesting that these proteins are bound to the inner membrane, presumably to localize AP1 in preparation for additional modifications and translocation to the periplasm. The cytoplasmic conversion of AP1 to AP2 is supported by the fact that AP2 is only released from an *ehp* mutant lacking a Group 3 gene.

The Group 3 gene products, EhpI, EhpJ, EhpK, and EhpL, collaborate to convert the red compound, AP2, to the orange compound, AP3. Data presented in Table 3.3 indicates that different Group 3 mutants release at least two and possibly three different red compounds that are crudely categorized as 'AP2' (Fig. 3.11). Thus, the products of the Group 1 and 2 genes synthesize a red compound that is subsequently modified in at least two different ways by the Group 3 gene products without altering the colour or hydrophobicity of the chromophore. As illustrated in Figures 3.10 and 3.11, EhpJKL appears to catalyze a reaction that creates a red AP2 derivative named AP2^{II} that is not amenable to conversion to AGA by Group 1 or 2 mutants. Of these gene products, EhpK is a putative oxidoreductase that could potentially alter the redox state of AP2 and perhaps lowering the reactivity of AP2 so that it cannot be taken up or converted to AGA by Group 1 or Group 2 mutants. This is supported by the observation that the product of EhpJKL, AP2^{II}, does not change colour on exposure to air, in contrast to AP2 / AP2^I, which are apparently more labile in the presence of oxygen. In contrast, EhpI either converts AP2 into a compound that can be modified to AGA by Group 4 gene products, or EhpI is not active in the absence of EhpJ or K or L. EhpI is thought to be a methyltransferase (Table 3.2) and as such is probably responsible for the only methyl group present in AGA (excluding the methyl group present in D-alanine), at position 9 of the phenazine nucleus (see Fig. 4.6). On this basis, it appears that EhpI and EhpJKL catalyze different reactions, that are carried out either simultaneously or sequentially (the EhpI reaction occurring first) since the EhpJKL modification results in a compound that cannot be converted to AGA by Group 3 and 4 gene products (Fig. 3.10). Alternatively, EhpI may alter the activity of EhpJKL rather than modify the structure of AP2; in this scenario the Group 3 gene products are likely to function as a single complex.

A red compound is also produced by *phzS* mutants of *P. aeruginosa* PAO1 (Mavrodi et al., 2001). Investigations in *E. coli* demonstrated that *phzM* gene is responsible for the conversion of PCA into this red pigment, which is further converted by PhzS into a blue phenazine compound, pyocyanin. Because the red product of PhzM is strongly hydrophilic it did not extract to solvents from culture supernatants and its structure has not yet been determined. Interestingly, the extraction of the red precursor AP2 / AP2^I from *EhehpJ* (O1) failed for the same reasons (no other mutants have been assessed, see Chapter 4). PhzM is predicted to be a methyltransferase responsible for the addition of a methyl group to the nitrogen at position 5 of the phenazine nucleus (see Fig. 4.6). Because AGA has a methyl group at position 9 of the phenazine nucleus, it is probable that the red phenazine intermediates isolated from mutants of Eh1087 and *P. aeruginosa* PAO1 are structurally different. Alternatively, the red product of PhzM could be the same as the red compound produced by *EhehpI*, if the conversion of AP1 to AP2 includes the addition of a methyl group to the nitrogen at position 5 of the phenazine nucleus and the function of *EhpI* is to subsequently transfer the methyl group to position 9 of the phenazine nucleus. It would be interesting to test these predictions by introducing *phzS* to an Eh1087 Group 3 mutant to see whether PhzS can convert AP2 to pyocyanin.

The second Group 3 gene product, *EhpJ*, is a transmembrane protein with similarity to protein families involved in the transport of a diverse range of compounds across membranes (Fig. 3.9). Taken with the findings that the Group 3 mutants release AP2, and that AP3 is found in culture supernatants of Eh1087, it is proposed that the mechanism by which AP2 is converted to AP3 includes the controlled export of AP3 by *EhpJ* (Fig. 3.11). Since AP2 is only released when any of the Group 3 gene products are missing, it is likely that these gene products also function together to prevent the premature release of AP2 from Eh1087. The lack of an *ehpJ* analog in or near PCA operons in *Pseudomonas* spp. indicates that the AP3 export mechanism in Eh1087 may be different to the phenazine-releasing mechanisms in *Pseudomonas* spp.

The conversion of AP3 to AGA is carried out by the products of the three Group 4 genes *ehpM*, *ehpN*, and *ehpO*, independently of all other *ehp* gene products (Figs. 3.4 and 3.12). The Group 4 gene products are also able to convert griseoluteic acid (GA, resulting from the loss of a D-alanine moiety by AGA), into AGA, and this reaction presumably occurs when Eh1087 grows in a moist environment, such as the stigma of blossoms. It is therefore possible that this reaction enables Eh1087 to convert hydrolyzed AGA back into active antibiotic on the stigma of blossoms where antibiosis is influential in the ability of Eh1087 to compete for niche colonization against the Fire Blight pathogen, *E. amylovora*. Although the exact mechanism by which AP3 is converted to AGA is not known, the conversion of GA to AGA by the Group 4

gene products strongly suggests that the mechanism involves a condensation reaction between AP3 or GA and D-alanine. Similarly, Yagishita (1960) showed that sonicated mycelia of *S. griseoluteus* possess an enzyme (or complex) that was capable of reacting glycolic acid and GA to create griseolutein A. Taken together, these findings indicate that the 6-hydroxymethyl group of GA may be commonly substituted with various side groups to produce different antibiotic active derivatives (Imamura et al., 1997; Sato et al., 1995; Singh et al., 1997; Yagishita, 1960).

How do EhpM, EhpN, and EhpO carry out this final antibiotic synthesis step? EhpM is a member of the AMP-dependent synthetase and ligase family of proteins. It shares greatest protein sequence similarity with the amino acid-binding domains of peptide synthetases such the threonine-binding SyrB1 protein involved in the synthesis of syringomycin by *Pseudomonas syringae* pv. *syringae* (Guenzi et al., 1998; Zhang et al., 1995). EhpM is therefore a good candidate to sequester and activate D-alanine for incorporation to AP3 in association with EhpN and EhpO. It is likely that EhpM specifically binds D-alanine, and that similar proteins in *Pelagibacter variabilis* may exclusively bind valine and glycine for the synthesis of valylgriseoluteic acid and glycylgriseoluteic acid, respectively (Imamura et al., 1997). Because EhpN is similar in sequence to acyl-carrier proteins, it is likely to juxtapose the sequestered D-alanine and AP3 (or GA) and thereby catalyze the condensation reaction between them. Investigations with *ehpO* mutants demonstrated that EhpO is also necessary for the conversion of AP3 to AGA, however its mechanism of action cannot be predicted at this stage.

Indirect evidence suggests that the conversion of AP3 to AGA takes place in the periplasm. Firstly, Figure 3.4A clearly illustrates that exogenous AP3 is more efficiently converted to AGA by Eh1087 mutants with functional Group 4 gene products than exogenous AP1, supporting the proposal that AP3 only needs to enter the periplasm for conversion to AGA, whereas AP1 would also have to cross the cytoplasmic membrane for conversion to AGA via AP2 and AP3. Secondly, no active antibiotic has been isolated from lysed Eh1087 cells, indicating that the final antibiotic synthesis step is unlikely to occur within the cytoplasm. Thirdly, the predicted translation sequence of the Group 4 gene product EhpM indicates that it has a transmembrane helix that would transverse the inner membrane to position ca. 80% of the protein in the periplasm, indicating that much of its activity is likely to be carried out within the periplasm (Fig 3.13). Finally, the release of a D-alanine moiety in the periplasm during the transpeptidation reaction that links peptidoglycan chains during synthesis of the bacterial cell wall would provide a natural reservoir of D-alanine for the final AGA-synthesis step.

Recombination deficient Eh1087 strains: A prerequisite for complementation analyses

In a previous investigation homologous recombination was reported to be a rare event, occurring at a frequency of 0 – 0.4 % (Kearns, 1993). However, in that study recombination was scored as cells that had lost both the plasmid, to become Tc^S, and the transposon insertion complemented by the plasmid, to become Km^S. This is an inadequate measure of recombination, as it relies on plasmid loss and cannot account for recombination without loss of the plasmid, which could be high given that the vector pLAFR3 was reported to be stable in Eh1087 in the same study. During the present study the true frequency of recombination was assessed by measuring the frequency that a plasmid containing the AGA gene cluster acquired a transposon insertion from an Eh1087 *ehp* mutant. By this methodology the frequency of marker exchange (via recombination) between cosmid pLA272 and Eh1087 mutants ranged from 1.3 % to 17 % for 24 h cultures. Thus, all complementation experiments involving Eh1087 mutants and cosmids have to be interpreted very carefully, given that recombination may have occurred in up to 17 % of the cosmid molecules after 24 h. Since RecA is absolutely necessary for homologous recombination through the pairing and exchange of homologous ssDNA (Kowalczykowski et al., 1994), a system was developed for the creation of Cm^R marked Eh1087 *recA* mutants (*recA777*) to prevent recombination during DNA complementation experiments. Recombination between pLA272 and *recA777* mutants of *EhehpA* or *EhehpC* was not detected. Since recombination in Eh1087 *recA777* mutants is, if anything, a very rare event (not detected in this investigation), plasmid integrity should be maintained during complementation analyses involving these strains.

The *recA777* allele exhibited dominance over *recA* in two ways. Firstly, once pL*ArecA777* was established in an Eh1087 strain, loss of the plasmid vector and maintenance of the *recA777* allele (Cm^R) was very rarely detected. Because of this, recombination was routinely carried out without selection for the plasmid vector. Secondly, during the creation of pL*ArecA777*, it was noted that pKS*recA777*, but not pKS*recA*, induced MMS sensitivity in *E. coli* MC4100 *recA*⁺. Studies of nearly identical RecA proteins from other bacteria indicate that the 95 aa missing from the C-terminus of the truncated Eh1087 RecA mutant protein (RecA777) are required for NTP binding, RecA monomer binding, and the alignment of RecA-filaments for exchange of complementary ssDNA strands (Kowalczykowski et al., 1994). The domains required to bind ssDNA (between residues 156-165 and 194-210) would be present in RecA777, and could enable this protein to bind ssDNA if sufficient RecA structure is maintained. RecA777 proteins bound to ssDNA would not be able to catalyze reactions that require NTP, such as denaturation of complementary strands, or LexA cleavage, and would effectively lower the concentration of

ssDNA not associated with RecA and inhibit the activity of normally functional RecA monomers. Similar effects were noted in *E. coli* in which truncated RecA proteins supplied in trans interfered with the activity of the native RecA protein by 'codominant inhibition' (Yarranton and Sedgewick, 1982). Observations like these lead to the proposition that RecA functions as a protein filament that can be influenced by the composition of its monomer components (Kowalczykowski et al., 1994). Such a scenario is attractive because it offers an explanation for the low frequency of recombination between established pL*ArecA777* cosmids and the Eh1087 genome. In such strains, there are more copies of the plasmid-encoded mutated *recA* gene than the single functional genomic *recA* gene, and therefore more potential inhibitors of RecA-mediated homologous recombination. Before pL*ArecA777* becomes established in a new host cell and *recA777* is expressed, functional native RecA proteins could carry out homologous recombination between the genomic *recA* and the introduced *recA777* without interference from RecA777.

Regulation within the AGA-gene cluster

The present study has uncovered many of the factors controlling expression of the AGA-gene cluster. Initial information was provided by gene predictions from DNA sequence, and the orientation of transposon insertions that formed active *lacZ* fusions to an AGA-biosynthesis gene demonstrated the orientation of genes as illustrated in Figure 3.5. A different feature of mini-Tn5*lacZ2* was utilized to find out information about the size and expression of operons within the AGA gene cluster using classical complementation experiments. mini-Tn5*lacZ2* possesses a kanamycin resistance gene under control of its own promoter and the transcript initiated by this promoter is not terminated within the transposon. Instead the process of transcription continues along the DNA into which the transposon has inserted. For this reason, transposon insertions in orientation 1 (O1) were aligned within the direction of transcription of the AGA-gene cluster; the *lacZ* gene of these insertions was under control of the gene into which the transposon had inserted, and all genes downstream of the insertion point were under the transcriptional control of the kanamycin resistance gene promoter in the transposon. Thus, orientation 2 (O2) insertions interrupted not only the gene into which the transposon had inserted, but all genes downstream that were under the same transcriptional control (i.e., part of the same operon) as the interrupted gene. The influence of this polarity is particularly notable in Figure 3.4, which illustrates how O1 mutants can express downstream genes and convert exogenously supplied intermediates into AGA, whereas O2 mutants can donate an intermediate, but lack the expression of one or more groups of genes required to convert an intermediate into AGA. The polarity of the O2 insertions was further utilized to determine the size(s) of the

operons within the AGA gene cluster. For instance, an Eh1087 *ehp* mutant is complemented by a plasmid containing a second copy of the AGA gene cluster in trans and therefore produces antibiotic. However, if the second copy of the gene cluster also contains a transposon insertion in a different gene, and no antibiotic is produced then the genes must be subject to transcriptional control by the same promoters; they are therefore part of the same operon, and vice versa. Furthermore, the amount of antibiotic produced by a mutant complemented in such a way offers a measure of the level by which the expression of one gene influences the expression of another, as illustrated in Fig 3.17.

The copy number of the introduced DNA relative to the copy number of the host DNA has an important influence on complementation. In the case of the AGA gene cluster this was complicated by the fact that it is located on a ca. 200 kbp plasmid of unknown copy number, although two lines of evidence suggest that the native Eh1087 plasmid is present in more than one copy per cell. Firstly, AGA-gene cluster insertions were isolated at a far higher frequency (1.7 %) during this investigation than would be expected for a 15.5 kbp region of DNA, assuming the chromosome of Eh1087 is similar in size to other *Enterobacteriaceae* (3 – 4 Mbp). Because insertions were spread evenly throughout the gene cluster region, and because auxotrophic mutants arose at ca. 1 %, it appears that mini-Tn5*lacZ2* inserted randomly throughout the Eh1087 genome. Two possibilities arise. Either the genome of EH1078 is ca. 880 kbp if the frequency of insertions to the AGA gene cluster is extrapolated, or, more plausibly, the genome size of Eh1087 is 3-4 Mbp and the plasmid on which the gene cluster is located is present at 3-5 copies per cell. A second finding that suggests the native plasmid is multicopy is the greater strength of signal from hybridizations to the gene cluster relative to chromosomal genes such as *rpoS* and *recA* (data not shown). Even so, the initial complementation results indicated pLAFR3 was present in more copies per cell than the native Eh1087 plasmid, since antibiotic production was lower when the gene that was limiting for antibiotic production was contained on the Eh1087 plasmid rather than the introduced, pLARF3-based plasmid (Fig. 3.16). Because complementation analyses were restricted to assessing the expression of genes within the gene cluster located on the native Eh1087 plasmid, they could only be used to determine the effect of a mutation on downstream genes, and not to differentiate between cis and trans acting factors.

The major finding of the gene cluster complementation experiments is that the gene cluster has an underlying operon structure that extends from *ehpA* to *ehpM*. Therefore all Groups are linked in cis. The expression of *ehpN* is independent of the Group 1, 2, and 3 genes, however it is influenced by expression of *ehpM*, which is dependent on expression of the upstream genes.

Within the *ehpA-ehpM* operon, exist alternative promoters for the Group 2 and Group 3 genes, and for *ehpK* and *ehpM* (Figs. 3.15, 3.17). These promoters probably have a very minor role in expression of the *ehp* genes since O2 mutants cannot sufficiently express genes downstream of the mutation point for conversion of AGA intermediates to AGA (Fig. 3.6). In fact, these promoters may only be active in the absence of expression of the overall *ehp* operon. If the *ehp* gene cluster is a mosaic of genes from various sources as predicted by contemporary evolutionary models (see below), these internal gene cluster promoters may be relicts of promoters that integrated into the gene cluster with a cognate gene or operon, but have since lost activity by mutation over time in the new environment of the gene cluster. This is particularly enticing given that each gene group appears to be preceded by independent, albeit weak, promoter. In summary, it is proposed that an overall operon structure is the most influential mechanism regulating transcription of the *ehp* gene cluster.

The finding that the AGA-gene cluster has an underlying operon structure provides an answer for an apparently anomalous observation of Kearns and Mahanty (1998) who found that plasmid pAH8, which contains all the Group 2 genes and most of the Group 3 genes, complemented what is now described as an *EhehpG* mutant poorly, unless pBE5b was also present *in trans* (Kearns, 1993; Kearns and Mahanty, 1998). The reason for this is now apparent; the transposon insertion in *ehpG* has a *cis*-acting polar effect on downstream genes, including the Group 4 genes, thus pAH8, which contains only the Group 2 and Group 3 genes, cannot complement *EhehpG*. However, sufficient genes are provided *in trans* by pAH8 (Group 2 and 3 genes) and pBE5b (Group 4 genes under control of the pBR322 Tc^R promoter) to complement the *EhehpG* mutant and create a strain that can produce AGA.

Complementation experiments could not differentiate between *cis* and *trans* acting regulatory influences, however indirect evidence from precursor-feeding experiments suggests that at least one *trans* acting factor may operate to control expression of the AGA-gene cluster. *EhehpJ* (O1) does not convert exogenous AP3 into AGA and therefore does not express the Group 4 genes, in contrast to *EhehpK* and *EhehpL* (O1) (Fig. 3.10). Since pBE5b confers on a cell the ability to convert AP3 to AGA in the absence of any other *ehp* gene (Fig. 3.12), the influence of *EhpJ* on the Group 4 gene products is not due to interactions between the *Ehp* proteins. Thus, in Eh1087, *ehpJ* must positively influence the expression of the Group 4 genes. This influence is presumably not necessary in a heterologous host containing pBE5b because the Group 4 genes are under the independent control of a Tc^R promoter present in the vector. Furthermore, the influence of *EhpJ* on the Group 4 genes must be *trans* acting rather than *cis* acting, as both *ehpK* and *ehpL* are expressed in *EhehpJ* (O1). Also, this influence is not observed for *EhehpK* or

EhehpL mutants in which *ehpJ* is active (Fig. 3.10). This proposition could be tested by inverting the Group 4 gene fragment in pBE5b so that the Group 4 genes are only expressed under control of their own promoter(s), creating a plasmid that should not enable a host cell to convert AP3 to AGA, if the prediction holds true, unless the regulatory influences of *ehpJ* are restored in trans.

The transcription of the majority of the AGA gene cluster as a single unit provided a means to assess whether or not regulatory influences acted at the level of translation. As shown in Figure 3.18, the expression of LacZ::Ehp fusion proteins varied from group to group and even within a group, indicating that each gene is subject to post-transcriptional regulation. Of the genes with active LacZ fusions, the expression of six gene products, EhpA, B, D, F, I, and L, was significantly (2 SD) reduced in stationary phase relative to log phase, whereas the expression of EhpC, G, and M did not drop during this transition (data not shown). The change in expression during entry to stationary phase offers a mechanism to explain the drop in antibiotic production after Eh1087 reached stationary phase (see Fig. 4.1). Perhaps more importantly, the expression of these genes may be rate limiting for antibiotic production, and therefore make a logical target for modification to enhance the amount of antibiotic produced by Eh1087 and to extend the period of antibiotic production. The production of antibiotic in growth-limited environments may improve the ability for Eh1087 to compete against *E. amylovora* that have already colonized a stigma, a feature that is currently lacking in Eh1087 and other potential Fire Blight control strains.

Differential gene expression can also account for the observation that antibiotic production by *E. coli* harboring the AGA-gene cluster was considerably lower than antibiotic production by Eh1087. Expression of the LacZ fusions to EhpA, F, I, and M, in *E. coli* demonstrated that expression of EhpA was only half that of Eh1087, whereas the expression of EhpF, I, and M, was only 10 – 20% that of Eh1087. Although the underlying transcription of the *ehp* gene cluster may be only reduced by half in *E. coli* as evidenced by 50 % *ehpA* expression, the positive regulatory influence(s) acting on the translation of the *ehpF*, *ehpI*, and *ehpM* transcripts in Eh1087 is (are) either missing or less active in *E. coli*. The lower expression of the *ehp* genes in *E. coli* relative to Eh1087 correlates well with the lower amount of AGA produced by *E. coli* relative to Eh1087. Another interesting finding from this experiment was that the expression of *ehpA*, *ehpF*, and *ehpI*, reduced during entry to stationary phase in Eh1087 but not in *E. coli* (Fig. 3.19). The simplest account for these observations is that a positive post-transcriptional regulatory mechanism specific to Eh1087 is turned off during stationary phase, and this is not seen in MC4100 because it does not possess this mechanism. Alternatively, a second negative

regulatory factor present in Eh1087, and missing in MC4100, may specifically down-regulate *ehp* gene expression in stationary phase.

A observation of Kearns (1993), considered in the context of what is now known about the AGA-gene cluster, suggests that the expression of *ehpR* may influence the expression of the AGA-biosynthesis genes. Kearns (1993) had noted that cosmid pLA255 fully complemented an *EhehpG* mutant, whereas pLA424 did not, in apparent contradiction of the fact that pLA424 possessed more of the AGA-gene cluster than pLA255 (Fig. 3.10). During the present study, DNA sequence was determined for the terminal section of the AGA-gene cluster cloned in each cosmid (data not shown). pLA255 was found to contain DNA downstream of bp 5404 (between *ehpD* and *ehpE*), and pLA424 possessed DNA downstream of bp 563 (interrupts *ehpR*). Since pLA424 could not complement an *ehpG* insertion mutant, it is probable that expression of *ehpR* affects expression of at least the Group 2 genes, perhaps via some mechanism that co-regulates AGA synthesis with expression of the resistance factor. In contrast, *ehpG* encoded by pLA255 is expressed, suggesting that any mechanism linking expression of *ehpR* with the biosynthesis genes is missing in pLA255, or that the section of the AGA-gene cluster cloned in this cosmid is artificially expressed under control of a vector promoter.

Resistance to AGA.

The translation product of a single gene, *ehpR*, divergently transcribed from the 15 *ehp* genes involved in AGA-synthesis, was found to confer AGA resistance on an otherwise AGA-sensitive host bacterium. For instance, *E. coli* MC4100 and *EhΔAGA* (*ehpR*-), which have similar AGA-sensitivity, are both transformed to be AGA-resistant by a plasmid encoding *ehpR*. *EhΔAGA* harbouring pBE5b can be induced to 'suicide' by supplying exogenous non-toxic AP3, which is converted into toxic AGA by the products of the Group 4 genes on plasmid pBE5b. Unlike Eh1087, *EhΔAGA* dies in the production of AGA as it is sensitive to the antibiotic (Fig. 3.12). These observations suggest that *ehpR* is necessary and sufficient for AGA-resistance both in an AGA-producing bacterium such as Eh1087, and in a heterologous host such as *E. coli*.

Three lines of evidence provide clues about the probable location of EhpR activity. Firstly, no intracellular antibiotic activity has been detected, secondly, the intracellular AGA intermediates AP1 and AP2 have no antibiotic activity, thirdly, conversion of AP3 to active AGA most likely takes place in the periplasm or on the outer membrane. On the basis of this data it is tentatively proposed that EhpR somehow prevents AGA from acting either on or inside the cytoplasmic

membrane. This is common theme amongst antibiotics, such as AGA, that are activated from inert precursors during export, presumably because it is energetically more efficient than drug uptake, inactivation, export and reactivation (Cundliffe, 1989). An alternative mode of resistance, alteration of the antibiotic target, is also unlikely on the grounds that this mode of action usually operates for antibiotics with single targets, and AGA is probably reactive against numerous targets (see Chapter 4) (Spratt, 1994). Thus, it is tempting to speculate that EhpR acts in a similar manner as the colicin V immunity protein, Cvi, which prevents periplasmically presented colicin V from re-entering the producing cell (Zhang et al., 1995).

The mode of action by which EhpR protects a cells from AGA is not fully understood, although some clues were provided by the degradation of AGA during exposure to bacteria. AGA degrades slowly in LB, but rapidly in LB containing bacteria, demonstrating that bacterial cells react with AGA (Fig. 3.20). However, the antibiotic activity of AGA does not detectably decrease during the first ten minutes of exposure to either Ea8862 or Ea8862 (pEhpR). Since many bacteria within a culture are killed or mortally wounded within 10 minutes of exposure to AGA (for instance, see Fig. 4.7), EhpR does not appear to inactivate AGA otherwise antibiotic activity would decrease immediately on exposure to Ea8862 (pEhpR). Additionally, the reaction between AGA and Ea8862 cells, which results in loss of antibiotic activity, is either not part of the killing process because it occurs whether bacteria die or not, or the reaction generates toxic by-products that EhpR protects against.

There are probably at least two different types of AGA-resistance, intrinsic AGA-resistance as displayed by *Pseudomonas aeruginosa* PA147-2, a bacteria isolated from the rhizosphere ($MLC_{AGA} > 16.0 \mu\text{g.ml}^{-1}$), and the dedicated resistance conferred by EhpR. When *ehpR* is absent, the rate of the reaction between AGA and a bacterial cell relates to the AGA-sensitivity of that cell, for instance PA147-2 is less reactive and more AGA-resistant than Ea8862. *ehpR* transforms Ea8862 bacteria into an AGA-resistant bacteria, but does not decrease the AGA-reactivity of these bacteria. This data supports the proposition that the resistance of PA147-2 involves poor recognition of AGA, whereas Ea8862 possesses outer membrane properties that enable it to recognize and react with AGA, perhaps as part of the uptake process, and EhpR presumably prevents further uptake of AGA by inhibiting translocation across the inner membrane. This hypothesis could be tested assessing the AGA reactivity of a large number of AGA-sensitive and resistant bacteria to see if intrinsic resistance always correlates with low reactivity. Furthermore, the activity of EhpR in a gram-positive bacteria would demonstrate whether or not it acts in the periplasm or outer membrane, as both are absent in these bacteria.

To ensure that sufficient EhpR is available to prevent self-poisoning of an AGA-producing cell, it is likely that expression of *ehpR* would be regulated in unison with the AGA synthesis genes. Within the 419 bp region between *ehpR* and *ehpA*, two potential *ehpR* promoters have been identified by sequence analysis that could initiate transcripts that converge and overlap with transcripts initiated by two potential *ehpA* promoters (promoter predictions scored the highest possible likelihood). Such an arrangement could offer a mechanism for co-regulation of the AGA-resistance and synthesis genes, similar to that of the well-characterized Mu phage system in which competition between the converging promoters PcM and PE is used to regulate the lytic and lysogenic pathways (Krause and Higgins, 1986).

A resistance mechanism may be associated with the PCA operon of *P. aureofaciens* 30-84. Pierson *et al.* (1992) reported that matings between *E. coli* and strain 30-84 were only successful in conditions in which phenazine production was suppressed, whereas *E. coli* harbouring the PCA operon could produce PCA and remain viable. However, the PCA resistance mechanism, or the gene(s) responsible for it, were not identified. Taken together with the fact that EhpR shows no similarity to known protein sequences or motifs, it appears that EhpR is a novel antibiotic resistance protein and that this is the first clear demonstration of a resistance gene associated with a phenazine antibiotic gene cluster.

Origin and features of the AGA gene cluster.

Recently, Mavrodi *et al.* (2001) detected by DNA-hybridization at least part of the PCA operon in phenazine-producing fluorescent *Pseudomonas* spp., but none of this operon was detected in several phenazine-producing isolates of *Burkholderia cepacia*, *Burkholderia phenazinium*, or *Brevibacterium iodinum*. The lack of similarity between the DNA sequence of the AGA gene cluster and the PCA operon found in *Pseudomonas* spp. demonstrates that although phenazine synthesis genes may not be detectable at the DNA level, the products of analogous or diverged genes may be similar, in sequence and function, and this may be the case in phenazine-producing bacteria that appear to lack the PCA operon. The lack of sequence similarity between the AGA gene cluster and the described PCA operons is at least partly due to the considerable difference in GC content between them, at 43.7 % for the AGA cluster, and 59.4 - 67.7 % for the PCA operons. This indicates that the AGA gene cluster is unlikely to have arisen in *Pseudomonas*, and that the relationship between the described *Pseudomonas* PCA operon and the AGA gene cluster is more likely to be analogous than homologous (Fitch, 2000). Furthermore, since the GC content of 21 described *Pantoea agglomerans* (syn. *E. herbicola*) strains is 55.1 – 56.8 %, the AGA gene cluster is unlikely to have originated in this species

(Gavini et al., 1989; Ochman et al., 2000). So what is the origin of the AGA gene cluster? It is tempting to speculate that the genetic basis for AGA production in Eh1087 and SANK 73794 originated in the same organism as the GC content of the AGA gene cluster falls within the GC content range of *Vibrio* spp. at 38-51 %, and AGA has only been isolated from Eh1087 and the marine *Vibrio* spp. SANK 73794 to date.

Currently, the origin of the AGA-gene cluster is inadequately estimated because it is based on comparisons with the DNA sequence of phenazine gene clusters characterized in only a handful of *Pseudomonas* species. The characterization of phenazine gene clusters in many other organisms is required to substantiate any evolutionary claims regarding their origin and relatedness. In addition, the characterization of phenazine gene clusters may provide useful data about the evolution of gene clusters in general. Recently, Lawrence and Roth (1996) proposed a 'selfish operon model of gene clustering' based on the fact that horizontal gene transfer is a potent evolutionary mechanism that enables gene clusters to be exposed to a large variety of hosts and environments. Therefore, even gene clusters with weak selective advantages are more likely to find a host in an environment that benefits from the gene cluster, than if the gene cluster was only transferred vertically. Since genes scattered around the chromosome cannot be transferred as a 'going concern' to a new host, they are not subject to the same selection pressure. Thus, the evolution of gene clusters able to provide a new host with a complete and functional metabolic pathway is favoured. On the basis of this theory, the AGA-gene cluster could have resulted from multiple gene clustering events. The observation that each gene Group is preceded by weak promoter activity suggests that each Group (see Table 3.1) may have been subclustered as separate operons prior to incorporation in the AGA gene cluster. For instance, the Group 1 genes, responsible for the synthesis of a PCA-like compound, may confer an advantage to a host in certain environments, an advantage sufficient for clustering of these genes. Presumably the addition of the Group 4 genes would have coincided with the resistance gene, to prevent death of the host in which the gene cluster evolved. In support of this the GC content of *ehpR* and *ehpMNO* are somewhat lower than the rest of the gene cluster, although the significance of this difference is unknown. Again, a measure of the relatedness of the AGA-gene cluster (or the subclusters within it) to phenazine genes in other organisms is required to test this proposition.

The complete AGA-gene cluster may be a horizontally-mobile element. Horizontally mobile genetic elements are ubiquitous in nature and are believed to have an important role in the evolution of bacteria (Miller, 1998; Ochman et al., 2000), and perhaps higher organisms (de la Cruz and Davies, 2000) although this is a source of conjecture (Kurland, 2000). Not only is the

AGA-gene cluster located on a plasmid (Kearns and Mahanty, 1998), which may itself be transmissible, but the cluster appears to be bordered by sequences with similarity to transposases and integrases (Fig. 3.21). As such the AGA-gene cluster has many (but not all) features of pathogenicity islands involved in the virulence of pathogenic bacteria (Hacker et al., 1997). Pathogenicity islands are normally associated with disease causing bacteria, however the physical features of these islands, as defined by Hacker et al (1997), have parallels in the AGA-gene cluster. The AGA-gene cluster is present in Eh1087 but not other species of *E. herbicola* (even from similar environments), it has different G+C content to the chromosome of Eh1087, it is a compact, distinct genetic unit, and it is bordered by potential 'mobility' genes. A key pathogenicity island feature that the AGA-gene cluster lacks is chromosomal location, although Mecsas and Straus (1996) suggested that location was irrelevant in view of the fact that genetic elements are in a constant state of flux. Eh1087 appears to be saprophytic, thus the AGA gene cluster cannot be termed a pathogenicity island per se, however as more AGA-type gene clusters are discovered it may become necessary to define a new set of 'islands', that are not directly involved in pathogenesis but have similar physical features to pathogenicity islands. In this regard, Hacker et al (1997) coined the term 'metabolic islands' to define pathogenicity-like islands involved in the physiology of microorganisms.

There is some evidence that suggests that the AGA-gene cluster is unique to Eh1087 amongst other *E. herbicola* isolates. Many New Zealand *E. herbicola* isolates possess plasmids similar in size to the native Eh1087 plasmid, however the genomic DNA of all twelve species that were tested did not hybridize to the AGA gene cluster. Since there is a good chance that at least some of these plasmids have a similar origin but the AGA-gene cluster is a feature specific to the Eh1087 plasmid, and since the GC content of the gene cluster is different to the Eh1087 genome, it is likely that the gene cluster has been acquired by horizontal transfer from some unknown source.

Plasmid maintenance often involves a mechanism termed post-segregational killing (PSK), in which plasmid-free daughter cells lack a plasmid-encoded antitoxin that provides protection against a diffusible plasmid-encoded toxin (recently reviewed by Gerdes, 2000). Thus, a gene cluster that is responsible for the production of an antibiotic and for resistance against that antibiotic could conceivably act as a PSK-system. The possibility that the AGA-gene cluster provided a mechanism for the stable maintenance of the native Eh1087 plasmid was crudely tested by assessing the maintenance of an AGA-gene cluster deletion derivative of this plasmid over three days in planta. The AGA-deletion plasmid was stably maintained in planta indicating that other plasmid-maintenance mechanisms must exist. Given that the AGA-gene cluster may

be a mobile element and therefore may be a recent addition to the native Eh1087 plasmid, it is not surprising that it is not required for plasmid maintenance.

Many of these propositions are highly speculative, but lead to interesting questions that warrant further investigation. Is the AGA-gene cluster part of a transposon or is it coincidentally located between two putative transposases? If it is part of a transposon, does it still function, and if so what induces it to jump? Is the plasmid containing the gene cluster mobilizable under certain conditions? And how was the AGA-gene cluster created in the first place? Investigations into the putative mobility genes at each end of the AGA gene cluster, and the ability of this gene cluster to be transferred to various hosts under a variety of conditions would provide useful data about the likely spread of the gene cluster in nature. Not only does this have relevance to the use of Eh1087 as a biological control agent, but perhaps more significantly it relates to the biology of 'metabolic islands' in general.

Chapter 4. CHARACTERIZATION OF THE ANTIBIOTIC PRODUCED BY *ERWINIA HERBICOLA* STRAIN EH1087

Introduction

Biological control agents operate via one or more of three general mechanisms; predation, competition, or the production of toxic chemicals. *E. herbicola* strains act invariably by competitively excluding *E. amylovora* from the stigmas of flowers (Johnson and Stockwell, 2000); for many strains the competitiveness of *E. herbicola* is enhanced by the production of antibiotics. Knowledge of the antibiotic structure and mode of action are necessary to fully understand the mechanism(s) involved in suppression of *E. amylovora* by candidate biological control strains, especially if these strains are to be deployed as biological control agents. This is particularly important in the United States, where the registration of microbial biological control agents requires the same rigorous testing as chemical agents. Characterization of biologically active natural products can also lead to the discovery of novel compounds and provide leads for the development of important new compounds with potential uses in agriculture and pharmacology (Powell, 1995).

In general, very little is known about the antibiotics produced by *E. herbicola*, which is surprising given their importance in *E. amylovora* suppression. A particularly well-characterized exception is pantocin B. Pantocin B and pantocin A and at least one other antibiotic are produced by Eh318 (Wright et al., 2001). The importance of antibiotic production by Eh318 in control of the Fire Blight pathogen was demonstrated in an immature pear fruit assay in which both Eh318 and crude antibiotic preparations from this strain suppressed the development of Fire Blight symptoms (Wodzinski et al., 1994). The genetic requirements for pantocin A and B synthesis are located in different regions of the Eh318 genome and a strain in which both genetic determinants are interrupted produces a third antibiotic, but it is the pantocins that are required for inhibition of *E. amylovora* (Wright et al., 2001). The chemical structure of pantocin B has been determined as (R)-N-[(S)-2-amino-propanoylamino)-methyl]-2-methanesulfonyl-succinamic acid (Brady et al., 1999; Sutton and Clardy, 2000). The structure of pantocin A is yet to be determined, possibly because pantocin B is a more potent inhibitor of *E. amylovora* and therefore of greater importance in biological control (Wodzinski et al., 1990). The targets of pantocin A and B are likely to be different as their antibiotic activity is

suppressed in the presence of excess histidine and arginine, respectively (Brady et al., 1999). Preliminary experiments demonstrated that pantocin B inhibited *N*-acetylornithine transaminase (Acorn) in the arginine pathway (Wodzinski et al., 1990). This was confirmed by Brady et al. (1999) who tested all arginine biosynthetic intermediates for suppression of antibiotic activity and found that no intermediates before *N*-acetylornithine inhibited by pantocin B activity. They further showed that pantocin B competes with *N*-acetylornithine, but not glutamine, to bind Acorn. Thus, pantocin B binds Acorn and prevents it from transferring an amine group from glutamine to *N*-acetylornithine during the synthesis of arginine. The activity of pantocin B has been further defined in a very recent study by Sutton et al (2001) who observed that pantocin B is similar to a tripeptide. They tested its activity in the presence of excess L-ala-gly-gly and found that it was reduced, presumably due to competition for uptake by a peptide transporter (Sutton and Clardy, 2001). To relate structural features of pantocin B to antibiotic activities, Sutton et al (2001) synthesized pantocin B variants and found that the stereochemistry at the N-terminal end influences uptake. They also demonstrated that the chemical structure of the central and C-terminal regions of pantocin B are highly specific for Acorn binding. Further characterization of pantocin B activity awaits the determination of the 3-D structure of Acorn.

Antibiotics from cultures of *E. herbicola* strains EhC9-1, Eh112Y, and Eh252 have shown suppressive activity against *E. amylovora* using overlay plate and pear slice assays (Hodges et al., 1980; Ishimaru et al., 1988; Vanneste et al., 1996). Unlike pantocin B, the detailed characterization of these antibiotics has not been published. A common property of antibiotics produced by *E. herbicola* is the inhibition of activity in the presence of particular amino acids, which has been used to differentiate between different strains of *E. herbicola* (Wodzinski and Paulin, 1994). Of the amino acids, histidine is most often inhibitory of the antibiotic activity of *E. herbicola* strains (Wodzinski and Paulin, 1994). Antibiotics that are ineffective in the presence of histidine include pantocin A, herbicolin O (but not herbicolin I) produced by EhC9-1, and a protease-sensitive antibiotic produced by Eh252 (Ishimaru et al., 1988; Vanneste et al., 1992). The activity of a second antibiotic produced by EhC9-1, herbicolin I, and the antibiotic produced by Eh112Y was reduced in the presence of yeast extract (Ishimaru et al., 1988). It is unclear whether antibiotic production in these strains is inhibited by yeast extract, or if one or more of the amino acid constituents of yeast extract negates the activity of these antibiotics.

Preliminary investigations into the antibiotic produced by Eh1087 indicated that it was different from antibiotics produced by other *E. herbicola* strains. In particular, antibiotic activity was not inhibited by essential amino acids unlike many *E. herbicola* antibiotics (Kearns, 1993; Kearns and Hale, 1996). The antibiotic was classified as bacteriocidal because no viable *E. amylovora*

were recovered from zones of inhibition surrounding colonies of Eh1087. The antibiotic activity in cell-free Eh1087 culture supernatants was not inactivated by proteases indicating that it is not a peptide antibiotic, or by exposure to pH 4.0 or 9.0 for one hour, and was able to pass through dialysis membrane (10 kDa cut-off) demonstrating that it was smaller than 10 kDa. Antibiotic activity was inactivated by 1 $\mu\text{g.ml}^{-1}$ Type 1 *Bacillus cereus* β -lactamase suggesting that the antibiotic possesses a β -lactam ring (Kearns and Hale, 1996). Further purification for characterization of the antibiotic was hampered by unreliable production in broth cultures (Kearns, 1993).

The overall objectives of the research reported in this chapter were to purify the antibiotic produced by Eh1087, determine its chemical structure, and characterize properties of the antibiotic with a view to elucidating its mode of action. Preliminary research involved maximizing the production of antibiotic, a problem noted in a previous investigation (Kearns, 1993). To optimize antibiotic yield, the time of antibiotic production and the influence of different carbon sources were assessed. Once conditions were established for reliable antibiotic production, a simple three stage purification process was developed. The chemical structure of the antibiotic was determined, as was the structure of an inactive compound that arose from storage of the antibiotic in aqueous solution. Chemical properties of the antibiotic and three precursors were compared with each other enabling predictions about the function(s) of the *ehp* gene groups. The antibiotic was subject to further investigations leading to speculation about its mode of action.

Methods

Extraction of AGA and precursor compounds.

Eh1087, *EhehpM*, or Eh Δ AGA (pBB7) were cultured in 500 ml of 1-A broth supplemented with 0.2 % glucose and 1 mM MgSO₄ in a 2 L Fernbach culture flask for 48 h (or 120 h for Eh Δ AGA(pBB7)) at 22 °C, with shaking at 200 rpm. Cells were removed by centrifugation at 15000 g for 10 min., and the culture supernatant was either extracted directly with 2 volumes of dichloromethane (DCM), or for Eh Δ AGA(pBB7) the broth was adjusted to pH 2.5 with HCl prior to extraction. The DCM extract was filtered through 0.45 μ M HA MF™-Membrane filters (Millipore) and dried by rotary evaporation. The soluble dried compounds were dissolved in 1-A salts adjusted to pH 2.5 with HCl. Insoluble material (predominantly GA) was removed by centrifugation, the media neutralized with 2M NaOH, and compounds that partitioned to chloroform were dried. Dried material was either dissolved in chloroform and stored at 4 °C, or dried onto 6 mm diameter filter disks (Schleicher & Schuell) for storage at ambient temperature. Compounds were separated and analyzed by C18 reverse-phase high-performance liquid chromatography (HPLC) by gradient elution in 10 - 100 % acetonitrile in water over 25 minutes using a Shimadzu SPD-M10A Diode Array Detector, SIL-10D Auto injector, and a SCL-10A Controller. To determine whether active antibiotic was present within Eh1087 cells, the cells removed from 1 L culture supernatant were rinsed with 5 ml fresh media, twice extracted with an equal volume of DCM, and the DCM fraction was concentrated by evaporation and assessed for antibiotic activity.

Antibiotic Susceptibility Tests

MIC and MLC determination by microbroth dilution was performed essentially as per the National Committee for Clinical Laboratory Standards (NCCLS) protocol as reported by Woods and Washington (1995). Microtitre wells containing 100 μ l LB medium with 5×10^5 cells, and antibiotic at log₂ serial dilutions, were incubated at the appropriate temperature for cell growth, and wells were visually assessed for cell growth after 24, 48 and 72 hr. LB medium was used, rather than Cation-Adjusted Mueller-Hinton Broth, to provide MIC and MLC values for organisms grown under standard laboratory conditions. Disk diffusion tests were also carried out as recommended by the NCCLS. An overnight culture of each strain to be tested was adjusted to a McFarland standard of 0.5 (ca. 1×10^8 CFU.ml⁻¹) and cross-streaked three times across the surface of an LB plate. Six mm filter disks containing one or ten μ g of the compound to be tested were placed on the surface of the lawn, plates were incubated under the appropriate conditions, and the diameter of the inhibition zone was measured. Plates containing obligate-

anaerobes were incubated in a two litre air-tight plastic container with a single AnaeroPack™•Anaero sachet (Mitsubishi Gas Chemical Co., Inc.). The removal of oxygen was confirmed using an Anaerobic indicator code no. BR 55 (Oxoid), and by comparing growth of obligate aerobes such as Ea8862 and *Pseudomonas aeruginosa*, to growth of the obligate anaerobe.

in vivo mutagenecity testing

Mutagenicity of AGA was assessed by measuring the frequency of *lacZ*- to *lacZ*+ reversions in a collection of *E. coli* tester strains containing different base substitutions at codon 461 of the *lacZ* gene. The assay was carried out as described by the strain developers (Ohta et al., 1998; Ohta et al., 1999), or with minor modifications (as outlined below) that did not affect the frequency of revertants when tested with known mutagens. The twelve *E. coli* tester strains are listed in Table A1, and the *lacZ* mutation in each strain is shown in Table 4.1. Each strain was grown overnight in MGT liquid medium (1 x VBE salts*, 0.5 % glucose, 50 µg.ml⁻¹ L-Tryptophan) supplemented with 10 µg.ml⁻¹ Ap for the WP3101P-WP3106P tester strains. Cells were collected by centrifugation, and resuspended in an equal volume of 10 mg.ml⁻¹ nutrient broth. 100 µl of each cell suspension (containing ca. 2 x 10⁸ CFU, 1 mg nutrient broth) was spread on an ML agar plate (1 x VBE salts^a, 0.5 % lactose, 1.5 % agar, 50 µg.ml⁻¹ L-Tryptophan) supplemented with various concentrations of the test compound, and incubated at 37 °C for 48 h. Data was scored as total number of *lacZ* revertants per plate.

Table 4.1. Base substitutions detected by *lacZ* reverter strains

Strain	Reversion event detected at <i>lacZ</i> codon Glu-461	Base substitution
WP3101	AAT TAG TCA → AAT GAG TCA (Stop → Glu)	A:T → C:G transversion
WP3102	AAT GGG TCA → AAT GAG TCA (Gly → Glu)	G:C → A:T transition
WP3103	AAT CAG TCA → AAT GAG TCA (Gln → Glu)	G:C → C:G transversion
WP3104	AAT GCG TCA → AAT GAG TCA (Ala → Glu)	G:C → T:A transversion
WP3105	AAT GTG TCA → AAT GAG TCA (Val → Glu)	A:T → T:A transversion
WP3106	AAT AAG TCA → AAT GAG TCA (Lys → Glu)	A:T → G:C transition

* 1 x VBE salts are composed of 1 mM MgSO₄, mM citric acid monohydrate, 57 mM K₂HPO₄, and 25 mM NaNH₄HPO₄

***in vitro* mutagenesis of pUC19 DNA by AGA.**

Ten micrograms of pUC19 dsDNA was exposed to four micrograms of AGA in 250 μ l phosphate buffer (pH 7.0) at 37 °C for 24 h and 72 h. Plasmid DNA was also treated with hydroxylamine (0.4 M) as a positive control for *in vitro* DNA mutagenesis. Five microgram samples were taken at both time points and purified by phenol-chloroform extraction, followed by ethanol precipitation and rinsing with 70 % ethanol. Purified DNA was introduced to DH5 α *recA*⁺ and *recA*⁻ cells prepared for DNA uptake by treatment with CaCl₂. Transformants were selected on LB supplemented with Ap and Xg.

Oxidation of hemoglobin by AGA.

AGA and AP3 were dissolved to saturation in 50 mM phosphate buffer (pH 7.4), and standardized to equivalent OD₃₆₄, usually 2.0 or 4.0 for a stock solution. Fresh red blood cells were separated from plasma by centrifugation (10000 g, 10 min., 4 °C), rinsed twice in 50 mM phosphate-buffered saline (PBS) (centrifuged at 500 g, 5 min., RT) and rinsed cells were lysed in 5 mM phosphate buffer (pH 7.4), and centrifuged for 3 min. at 9250 g to remove solid material. The red blood cell lysate was equilibrated to an OD₅₇₇ of 0.5 - 0.7 before being exposed to standardized AGA or AP3 at 37 °C. At 0, 120, 240 and 360 min, 100 μ l samples were taken and diluted 1:10 in phosphate buffer before the absorbance of the solution at 560, 577, 630 and 700 nm was measured to calculate the change in redox state of hemoglobin. Additionally, at each time point A₃₀₆ and A₃₆₄ were measured and the difference between them gave a measure of the concentration of AGA and AP3. One mM acetylphenylhydrazine (APH) was used as a positive control for hemoglobin oxidation, and reactions were carried out in the presence or absence of azide to inhibit catalase. The concentration of oxyhemoglobin and the oxidized derivatives methemoglobin and hemichrome were derived from the spectral measurements as described previously (Winterbourn, 1985). Van den Berg modifications of these equations were used when it was apparent that ferryl-Hb (methemoglobin-H₂O₂ complexes) may have been forming (C. Winterbourn, pers. comm.).

Table 4.2. Equations to calculate the concentration of haemoglobin oxidation products

Original Winterbourne Equations			
[Oxyhemoglobin]	=	119A ₅₇₇ - 39A ₆₃₀ - 89A ₅₆₀	
[Methemoglobin]	=	28A ₅₇₇ + 307A ₆₃₀ - 55A ₅₆₀	
[Hemichrome]	=	-133A ₅₇₇ - 114A ₆₃₀ + 233A ₅₆₀	
Van den Berg modification using correct figures of Whitburn (for H ₂ O ₂ adducts)			
[Oxyhemoglobin]	=	536A ₅₇₇ - 104A ₆₃₀ - 472A ₅₆₀	
[Methemoglobin]	=	430A ₅₇₇ + 1500A ₆₃₀ - 784A ₅₆₀	
[Ferry-HB]	=	-536A ₅₇₇ - 476A ₆₃₀ + 976A ₅₆₀	

Reduction of cytochrome C by AGA

The redox activity of AGA was assessed *in vitro* by measuring the reduction of cytochrome C in the presence of NADPH. Reactions containing one or more of the components listed in Table 4.3 were made up to 1 ml with 50 mM phosphate buffer (pH 7.4), and the rate of cytochrome C reduction in $\mu\text{M} \cdot \text{min}^{-1}$ was calculated by measuring the change in A₅₅₀ over 3-5 min. at 25 °C, where production of Cyt.C_{red} = slope / ϵ , and ϵ (Cyt.C_{red}) = 21100 M.cm⁻¹. All chemicals except AGA and AP3 were purchased from Sigma Chemical Co..

Table 4.3. Components of cytochrome C reduction reactions

Component	Stock conc.	Working Conc.
Cytochrome C	2 mM	20 μM (est.)
AGA	saturated	0.5, 1.0, 2.0 $\mu\text{g} \cdot \text{ml}^{-1}$
AP3/GA	saturated	0.5, 1.0, 2.0 $\mu\text{g} \cdot \text{ml}^{-1}$
NADPH	10 mM	50, 100 μM
NADH	10 mM	50, 100 μM
NADPH Reductase	2 U.ml ⁻¹	0.02 U.ml ⁻¹
Superoxide Dismutase	30 mg.ml ⁻¹	300 $\mu\text{g} \cdot \text{ml}^{-1}$
Catalase	35 mg.ml ⁻¹	350 $\mu\text{g} \cdot \text{ml}^{-1}$
Menadione	20 mM	200 μM

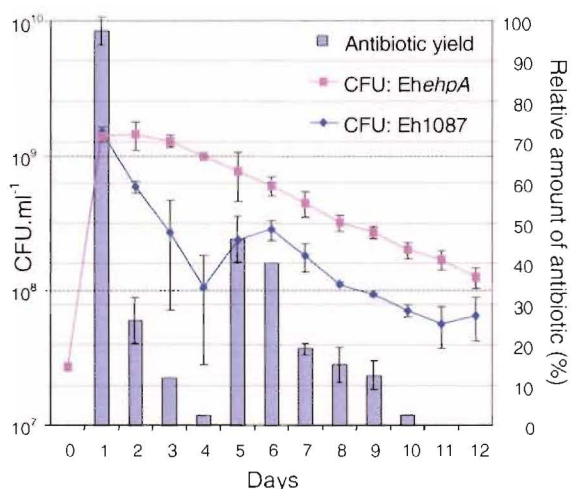
Results

4.1. Optimization of antibiotic yield from Eh1087 cultures

4.1.1. The influence of growth phase on antibiotic production by Eh1087

To determine the optimal time for antibiotic extraction in small scale cultures, the production of antibiotic by Eh1087 cultured in minimal-1A medium was quantified over twelve days (Fig 4.1). These data demonstrate that Eh1087 produces maximum antibiotic as the growth media becomes exhausted, after which time antibiotic activity decreases to barely detectable levels and the Eh1087 population drops dramatically. After five days the population of Eh1087 recovers and antibiotic activity is partially restored. To determine whether the antibiotic was involved in the changing population size of Eh1087 over time, similar data was collected for *EhehpA* (AGA-) (Fig. 4.1). Because *EhehpA* does not exhibit these same population dynamics, it is likely that either the metabolic load of antibiotic production, or the activity of the antibiotic compound, causes the Eh1087 population to decline. Since the yield of antibiotic from Eh1087 cultures was found to be greatest at the first sampling point, 24 h after the inoculation of fresh media, it was pertinent to check that greater antibiotic yields could not be achieved in less than 24 h. In order to assess this, cultures of Eh1087 were sampled for antibiotic activity and population size at hourly intervals (Fig. 4.2). This experiment showed that although antibiotic is produced by Eh1087 soon after subculturing to ca. 10^6 cells.ml⁻¹, the best yield per culture volume was achieved when cultures entered stationary phase.

Figure 4.1. Antibiotic yield and population size of Eh1087 cultures over time



Antibiotic was partitioned from the supernatant of Eh1087 cultures to chloroform, dried to maintain activity, and all samples were assessed for relative activity on the same lawn of Ea8862. No antibiotic was obtained from *EhehpA* cultures. Population sizes of both strains were determined by dilution series. Data is the average of two replicates and error bars represent 2 standard deviations.

4.1.2. Influence of carbon / energy source on antibiotic production by Eh1087

AGA production by Eh1087 cultured in minimal medium with glucose as a sole carbon source was compared with sucrose and fructose because these sugars are reported to be the most common sugars found in nectar, and therefore their presence in blossoms was thought to be relevant to the production of AGA by Eh1087 in planta (Fig. 4.2). Eh1087 produced more antibiotic on glucose than other carbon sources, and the timing of production varied between each medium. Compared to minimal medium, in rich medium (LB) antibiotic production appeared to be both delayed and reduced. Antibiotic production by Eh1087 did not vary detectably when grown on minimal medium agar plates containing a range of glucose concentrations from 0.1 % to 2.0 %. On the basis of these findings Eh1087 was routinely cultured in 1-A with glucose as the sole carbon source to prepare antibiotic for purification and further analyses.

4.2. Purification of antibiotic from Eh1087 cultures

4.2.1. Solvent extraction of Eh1087 culture supernatants

Evidence presented in chapter 3 suggested that Eh1087 produces a phenazine antibiotic (Table 3.1). Since the first step in phenazine isolation from the culture supernatant of other organisms commonly involves extraction with a solvent such as chloroform (Byng and Turner, 1976; Chang and Blackwood, 1969; Gerber, 1969), this was attempted for Eh1087 cultures. The cell-free supernatant of Eh1087 cultures was extracted with chloroform, the solvent phase was collected and dried. The dried compounds were redissolved in sterile media and assessed for antibiotic activity. Although there was variation in the extraction of antibiotic from one culture to the next, up to 80-90 % of the antibiotic activity partitioned to the chloroform phase (Fig. 4.3). This could be increased by further chloroform extractions. The phenazines that have been described in the literature are usually coloured and possess two absorbance maxima, between 250 - 290 nm and 350 – 400 nm (Turner and Messenger, 1986). The crude chloroform extract from Eh1087 culture supernatant was yellow, and possessed a strong absorbance maximum at 264 nm and weaker maximum at 364 nm, providing the first experimental evidence that Eh1087 produces a phenazine antibiotic.

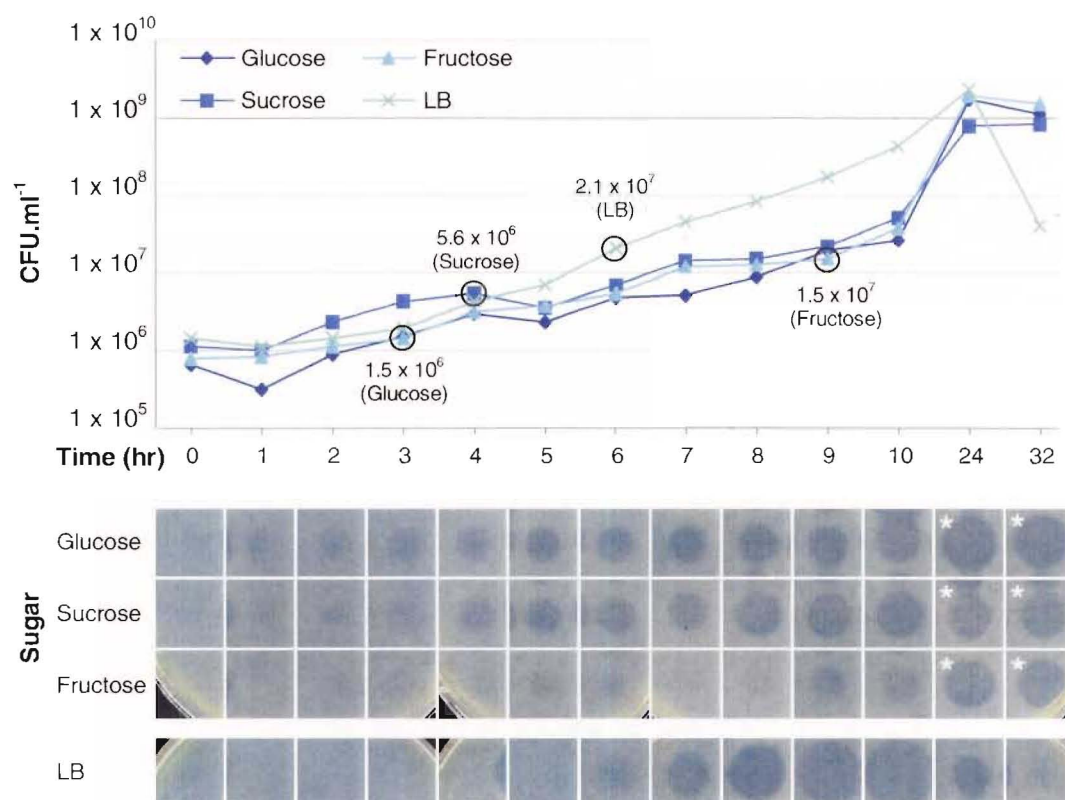


Figure 4.2. Influence of growth phase and media composition on antibiotic production

Eh1087 was grown overnight in LB or 1-A minimal media supplemented with 0.2 % glucose, sucrose, or fructose, and subcultured to the same medium to ca. 10⁶ cfu.ml⁻¹. At each time point, 500 µl samples were taken for cfu.ml⁻¹ enumeration and antibiotic extraction. Antibiotic was usually extracted from 100 µl of culture supernatant, however samples marked with a white asterisk show antibiotic extracted from 10 µl of culture supernatant. Samples were taken every h for 10 h, and again at 24 and 32 h. The population size of *Eh1087* grown in each media type (colour-coded) is shown at top, and the relative antibiotic activity of each culture is shown below as clearings in a lawn of *Ea8862*. Circled population sample points indicate the approximate time when antibiotic production is similar in each media type; the figure next to each circle is the population size at that sampling point.

4.2.2. Secondary purification by acid solubilization

In an earlier report the activity of the Eh1087 antibiotic was retained after exposure to pH 4.0 and 9.0 for 30 min. (Kearns and Hale, 1996). In the present study the activity of the antibiotic produced by Eh1087 was found to be maintained in acidic conditions but to rapidly decrease above pH 9.5 within 30 min (Fig. 4.3). Activity also decreased over a number of hours at neutral pH and the decrease was more rapid in basic conditions. Figure 4.3 also illustrates how antibiotic activity only partitions into chloroform from aqueous solutions that are near neutral pH. When the chloroform extract was dried and redissolved in 1-A media at pH 2.5, only 10-20 % of the material dissolved, whereas at pH 7.0 nearly all material dissolved. However, when the acidified solution was neutralized with NaOH, re-extracted to chloroform and antibiotic activity assessed, it was found that nearly all antibiotic activity was retained. This simple procedure separated AGA from nearly all other compounds, particularly AP3, which is a major component of chloroform extracts from Eh1087 culture supernatants (Fig. 4.4C, panels 1 and 4).

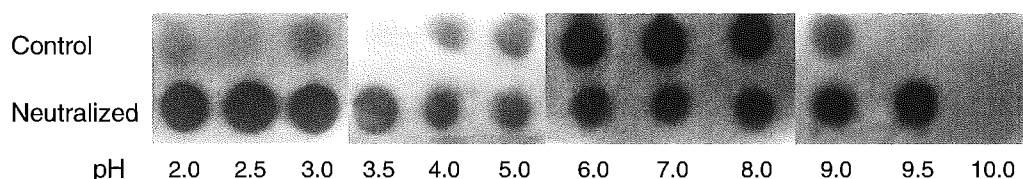


Figure 4.3. The pH sensitivity and chloroform extractability of AGA

The differential partitioning of antibiotic from pH-adjusted solutions to chloroform (control) or from neutralized solutions to chloroform. 40 μ g of crude chloroform-extracted material from Eh1087 culture supernatant was redissolved in 200 μ l 1-A medium adjusted to a pH between 2.0 and 12.0, and maintained for 30 min at room temperature. The dissolved material was transferred to a new container and either directly extracted with 200 μ l chloroform (control) or neutralized before extraction with chloroform. Antibiotic compound(s) only transferred to chloroform from aqueous solution near neutral pH. Although only ca. 10 - 20 % of the material dissolved below pH 4.0, full antibiotic activity was retained. Of the original 40 μ g material per treatment, eight μ g was assessed for antibiotic activity on a lawns of Ea8862. Photographs of the appropriate section of each lawn were aligned using Photoshop 5.0 and although there is some variation in lawn sensitivity to the extracted antibiotic, the overall trend is clear.

4.2.3. Final antibiotic purification by LH-20 size exclusion chromatography

The proportion of non-antibiotic compounds present after acid solubilization of the antibiotic was variable. This contamination was subsequently shown to be predominantly griseoluteic acid (GA) formed by hydrolysis of the antibiotic during extraction in aqueous buffer (Figure 4.4B shows an extraction in which an standard amount of GA was present). Therefore a third purification step was used in which the acid-soluble compounds were extracted to chloroform and fractionated through a Sephadex LH-20 column. Two yellow fractions eluted at nearly the same time from the column, and were followed some time later by GA, which formed a red precipitate when dried. The first yellow compound had ca. 2.5 % of the antibiotic activity of the second. Furthermore, because mass spectrometry data indicated that the first yellow compound to elute from the column was likely to be composed of antibiotic-detergent adducts, it was discarded (Y. Feng, pers. comm.). The second yellow compound was sufficiently pure for 1D and 2D nuclear magnetic resonance spectroscopy and mass spectrometry.

4.2.4. Properties of the purified antibiotic

As expected, the relative activity by weight of the purified compounds increased with each step of the purification process (for example see Fig. 4.4A). Purification was also demonstrated by HPLC analyses of the material present at each stage of the purification process (Fig. 4.4C). Analysis of the HPLC fractions demonstrated that a compound eluted at 6.9 minutes with antibiotic activity, in contrast to AP3 (10.7 min.) and GA (11.9 min.) (Fig. 4.4B). Subsequent discussions with S. Devenish (Dept. Chemistry, University of Canterbury, NZ) suggest that such comparisons must be judged carefully since the HPLC machine used in these studies was known to have leaking valves that could compromise the reproducibility of one run relative to the next. Antibiotic was purified from many Eh1087 cultures using this three-step process, which was sufficient to isolate antibiotic with the same activity and absorbance (see below) per weight on each occasion. Because dried antibiotic did not lose activity over three days when exposed to -20 °C, 4 °C, 18 °C, 30 °C, 37 °C or 65 °C, but lost nearly all activity within an hour in water at 65 °C, the antibiotic compound was routinely stored dried in a desiccator at ambient temperature.

Using this three-step purification process, approximately one milligram of antibiotic was obtained from a one litre Eh1087 culture grown in minimal medium. Antibiotic activity could be detected from as little as 50 ng AGA per 6 mm diameter filter disk on a lawn of Ea8862. Antibiotic dissolved in minimal media or water has absorbance maxima at 262 and 362; at a concentration of 10 $\mu\text{g}.\text{ml}^{-1}$ an antibiotic solution has an OD_{262} of ca. 0.53 - 0.54.

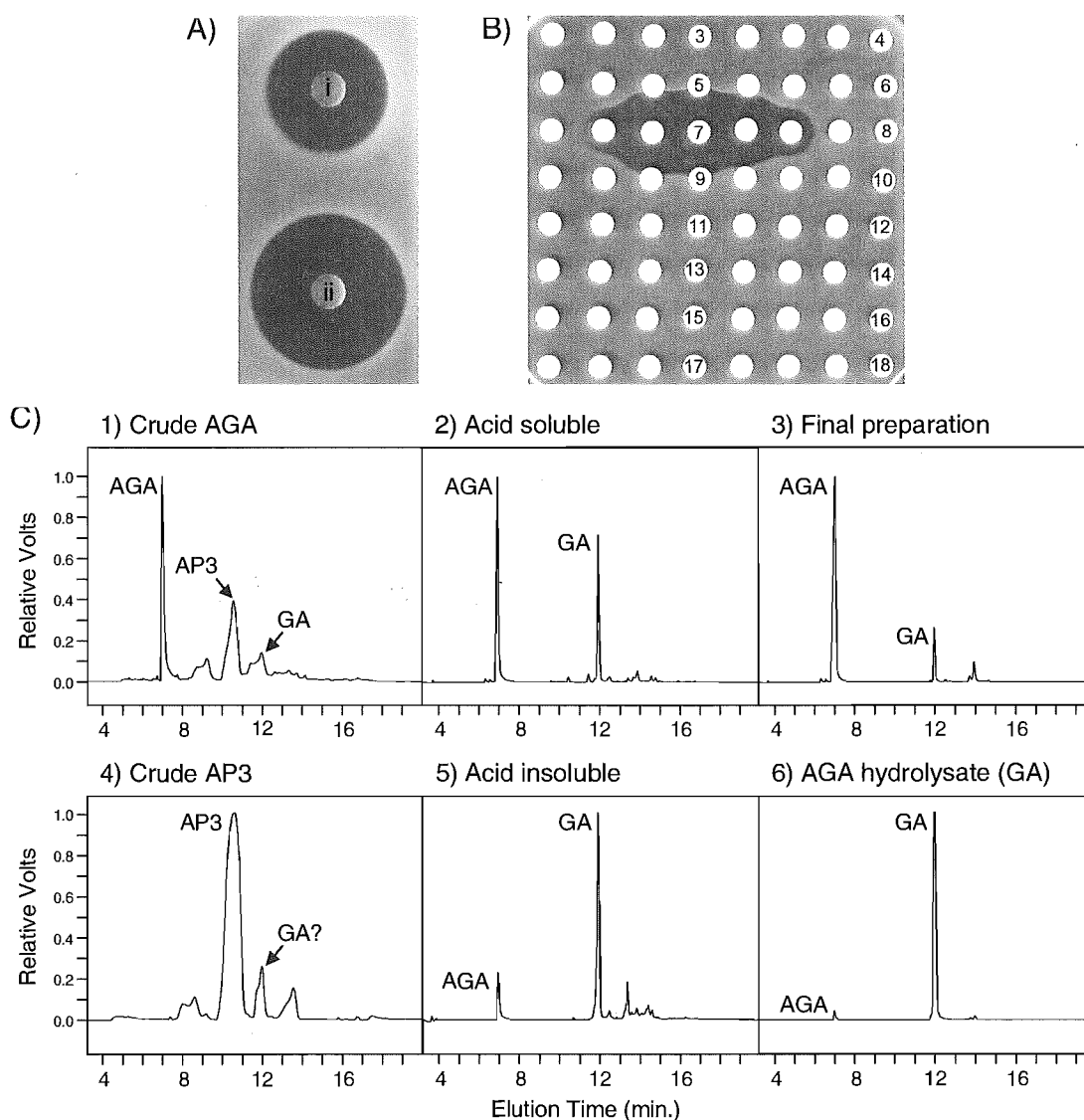


Figure 4.4. The purification of AGA from minimal media cultures of Eh1087

A) Antibiotic activity of 6 mm filter disks containing either (i) 10 μ g crude culture supernatant extract or (ii) 10 μ g of material after acid-solubility purification. B) Assessment of antibiotic activity for samples eluting at the indicated times (min) from a water / acetonitrile gradient; samples start at 2.25 min and go from left to right. Samples were collected every 25 s, dried, redissolved in chloroform and absorbed onto six mm filter disks. Fractions tested for antibiotic activity correspond to the HPLC traces shown in part (C). C) HPLC traces for the antibiotic preparations listed in part (A), and for (4) antibiotic precursor 3 (AP3) extracted from the culture supernatant of *EhehpN*, (5) the acid insoluble material, and (6) the phenazine compound, Griseoluteic acid (GA), resulting from hydrolysis of AGA.

4.2.5. Properties of the coloured AGA intermediates

To compare the coloured intermediates produced by different *Ehehp* mutants, the extraction of all coloured intermediates from culture supernatant to chloroform was attempted. AP1 and AP3 partitioned to chloroform, whereas AP2 did not, indicating that AP2 is more polar than AP1, AP3 and AGA (the antibiotic was found to be D-alanylgriseoliteic acid (AGA) as described in section 4.3.2). Since AP1 is released in very small amounts from Group 2 mutants, it is likely to be converted to AP2 intracellularly. Therefore, AP1 was extracted from cultures of *EhΔAGA* (pBB7) which enhanced the yield of AP1 because pBB7 is a multicopy plasmid that contains the Group 1 genes *ehpA-ehpE*. AP1 could be extracted to chloroform from the culture supernatant in which this strain had grown only after the medium was acidified, in a similar manner to the isolation of phenazine-1-carboxylic acid (PCA) from the culture supernatants of by *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 (Thomashow et al., 1990). Since AP2 could not be extracted from culture supernatant to chloroform comparison with AGA, AP1 AP3 and PCA was not possible. The final precursor (AP3) was isolated from a culture of the Group 4 mutant, *EhehpN*. HPLC analysis was used to compare AP1, AP3, AGA and PCA (Fig. 4.5). The different elution times of these compounds indicate that they have different solubility in water and confirms that they are different molecules (Fig. 4.5A). However, all four compounds possessed absorbance spectra with characteristics common to phenazines (Fig. 4.5B) indicating that they all possess the phenazine nucleus (Turner and Messenger, 1986). Notably AP3 and AGA possessed similar absorbance spectra and λ_{\max} , whilst AP1 and PCA have very similar absorbance spectra and the same λ_{\max} . Thus, AP1 and PCA are probably more closely related to each other than to AP3 or AGA.

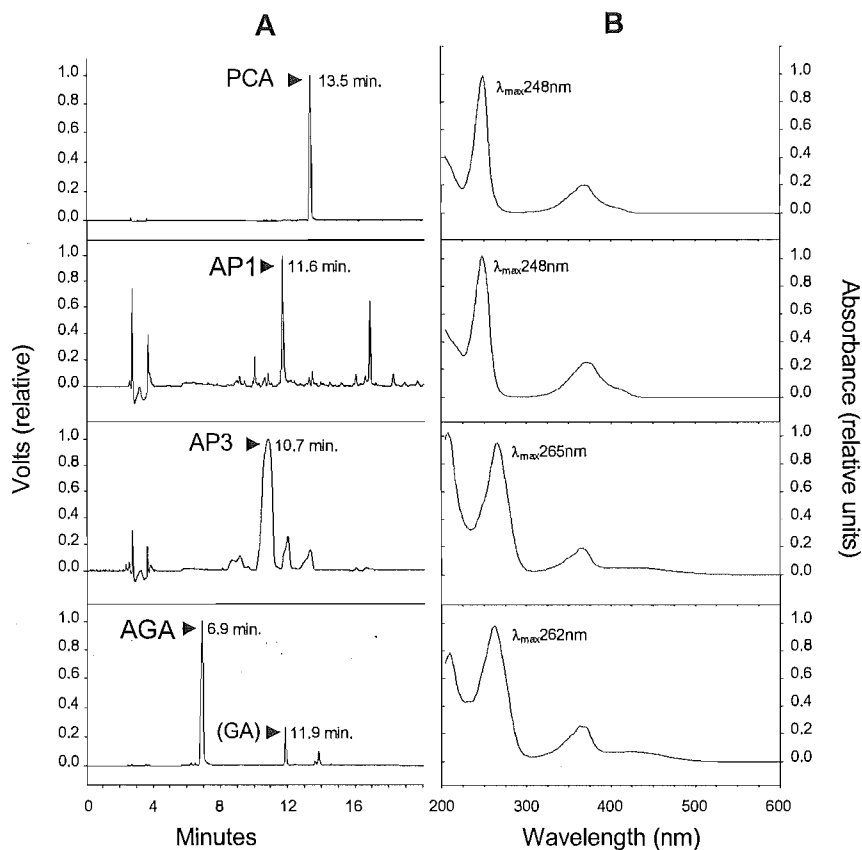


Figure 4.5 Comparison of PCA, AP1, AP3, and AGA

Comparison of solubility and UV-visible absorbance spectra of phenazine-1-carboxylic acid (PCA), AGA precursors AP1 and AP3, and AGA separated on a water / acetonitrile gradient by C_{18} reverse-phase HPLC.

A) Chromatograms illustrate the different elution times for each compound (measured at 265 nm). For the chromatograms of PCA, AP1, and AP3 only the compound(s) responsible for the dominant peak (indicated) have the absorbance spectra common to phenazines (Turner and Messenger, 1986). The small peak at 11.9 minutes in the AGA chromatogram also has phenazine characteristics and was found to be GA (see section 4.3.1).

B) Comparison of the ultraviolet-visible spectra for the major peak in each chromatogram.

4.3. Structure of the antibiotic produced by Eh1087

4.3.1. Structure of the antibiotic-breakdown product

Purified antibiotic is yellow and breaks down in water or alcohol to a red compound that possessed no detectable antibiotic activity against Ea8862. Eventually it was found that the active yellow compound was stable in chloroform, however before this was known the structure of the red compound was determined as it was expected to provide clues about the structure of the active compound. The structure of the inactive red compound was elucidated as griseoluteic acid (GA) by nuclear magnetic resonance (NMR) and mass spectrometry. All data were obtained and interpreted by Yunjiang Feng, Department of Chemistry, University of Canterbury. GA was the first known phenazine compound to be isolated from Eh1087, and indeed any *Erwinia* or *Pantoea* spp. as far as the author is aware (Fig. 4.6). The breakdown of the Eh1087 antibiotic into GA strongly suggested that the active compound was also a phenazine, with side group(s) that are easily hydrolysed in aqueous solution.

4.3.2. The chemical structure of AGA

The finding that the Eh1087 antibiotic was stable in chloroform enabled the structure to be determined by 1D and 2D NMR and mass spectrometry. NMR data are presented in Appendix A4 for completeness. The antibiotic produced by Eh1087 was indeed related to GA, but possessed an alanyl ester at position 6 (Fig. 4.6). The stereochemistry of the alanine group was determined by derivatizing an AGA hydrolysate with 1-fluoro-2,4-dinitro-5yl-L-alanine amide (FDAA), and analysing the derivatives by LC-MS relative to FDAA derivatives of D-alanine and L-alanine (Y. Feng., pers comm.). This experiment demonstrated that hydrolysis of the Eh1087 antibiotic released D-alanine, and therefore the antibiotic was D-alanylgriseoluteic acid (AGA). Interestingly, D-alanine is an amino acid found almost exclusively in bacteria where it forms a key component of the cell wall. The chemical structures of AGA and GA indicate that GA could easily arise by hydrolytic cleavage of the D-alanyl moiety from AGA (see also section 3.4.2). To determine the concentration of purified AGA from spectral data, the molar extinction coefficient (ϵ) was estimated from the absorbance (0.54) of a $10\text{ }\mu\text{g.ml}^{-1}$ solution of AGA (Mw 355.35) in water: D-alanylgriseoluteic acid, λ_{max} 262 nm ($\epsilon = 19200$). Therefore, the concentration of a solution of AGA in water with an absorbance of 1.0 at 262 nm is ca. $52\text{ }\mu\text{M}$ ($18.5\text{ }\mu\text{g.ml}^{-1}$).

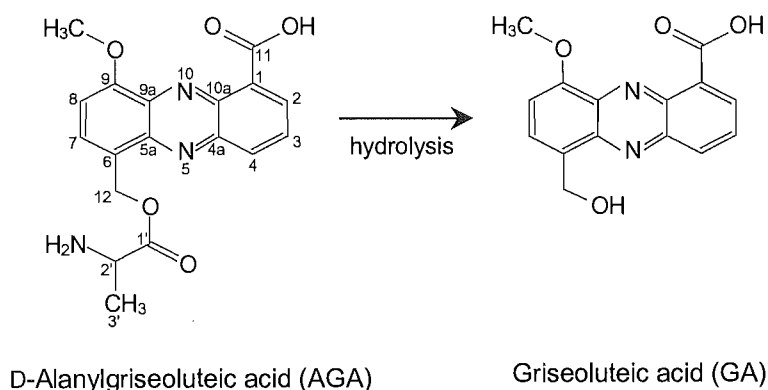


Figure 4.6. The chemical structures of AGA and GA

4.4. Spectrum of AGA-susceptible organisms

Once AGA was found to have no detectable impurities (by NMR analysis), the minimal concentrations required for inhibition and killing (MIC and MLC, respectively) of various organisms could be measured. It was anticipated that commonality amongst organisms with similar susceptibility to AGA could provide clues about antibiotic activity. To measure MIC and MLC, bacteria were cultured in microtitre dish wells containing LB media supplemented with a log₂ dilution series of AGA. In broth dilution, no antibiotic activity was retained after 48 h. Thus, a dilution series could be scored at 72 h for MLC since any bacteria that had survived the initial AGA exposure would repopulate the media by 72 h. MIC values were often the same or a single dilution lower than MLC values (for example see Table 4.4). The broth dilution method could be used to quantify antibiotic activity measured by the disk diffusion method, a simpler technique which involves measuring the diameter of a killing zone on a lawn of sensitive bacteria. Comparison between the broth dilution method and the disk diffusion method indicated that the latter was an accurate and reproducible method for estimating the MIC for a particular organism.

The MIC of many of the strains used throughout this investigation was assessed and this data is presented in the relevant sections. The MIC values for six *E. coli* strains are presented in Table 4.4 because they demonstrate i) the variation in AGA-susceptibility amongst strains of the same

species, and ii) that MIC values can indicate general trends, in this case that *recA* mutants are more sensitive to AGA than isogenic *recA*⁺ strains. For a collection of different bacteria with MIC values ranging from 0.2 to 16 $\mu\text{g.ml}^{-1}$, a strong correlation was noted between the measured MIC and the halo size of a filter disk containing AGA on a lawn of the test organism. Subsequently, a range of Gram⁺ and Gram⁻ organisms were tested using the disk diffusion assay, and estimated MIC values are reported in Table 4.5 (strains and technical expertise were kindly provided by David Bean, Dept. Plant and Microbial Sciences, University of Canterbury). The cutoff point for insensitive organisms was arbitrarily chosen as $\geq 8.0 \mu\text{g.ml}^{-1}$, because disks containing 10 μg AGA either produce no clearings, or very small clearings (7 - 8 mm diameter, where 7 mm is the detection limit for 6 mm disks), on organisms with this level of sensitivity. Ten μg disks produce substantial clearings on sensitive organisms (10 - 36 mm diameter), however it should be noted that this is not a clinically relevant measure of sensitivity as described by Woods and Washington (1995). Both Gram⁺ and Gram⁻ bacteria exhibited a range of AGA-sensitivities. The data reported in Table 4.5 also demonstrate that AGA is active in an micro-anaerobic environment as it has good activity against the obligate anaerobes, *Bacteriodes fragilis*, and *Clostridium perfringens* cultured anaerobically. In summary, it is clear that there is no obvious rule by which the sensitivity of bacteria to AGA can be predicted; this data has to be determined empirically for each strain.

Table 4.4. Sensitivity of *recA*⁺ and *recA*⁻ *E. coli* to AGA

<i>E. coli</i> strain	MIC		MLC	
	<i>recA</i> ⁺	<i>recA</i> ⁻	<i>recA</i> ⁺	<i>recA</i> ⁻
DH5 α	1.6	0.8	1.6	1.6
MC4100	3.2	0.4	3.2	0.8
DE880	0.8	-	0.8	-
DE1491	-	0.4	-	0.4

MIC and MLC data were determined by the broth dilution method

Table 4.5. Screen of gram- and gram+ bacteria for AGA sensitivity.

Sensitive Organisms (NZRM#)	Gram -/+	¹ Zone	Est. MIC
<i>Arcanobacterium haemolyticum</i> (816)	+	31	<0.2
<i>Bacillus subtilis</i> (698)	+	18	0.8
<i>Branhamella catarrhalis</i> (2565)	–	23	0.4
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> (3242)	–	20	0.4
<i>Corynebacterium diphtheriae</i> (23)	+	18	0.8
<i>Escherichia coli</i> (916)	–	15	1.2
<i>Haemophilus influenzae</i> (3315)	–	30	0.2
<i>Helicobacter pylori</i> (8823)	–	36	0.2
<i>Neisseria gonorrhoeae</i> (1036)	–	31	0.2
<i>Neisseria lactamica</i> (2590)	–	25	0.2
<i>Shigella flexneri</i> (3476)	–	20	0.4
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> (1056)	+	22	0.4
<i>Streptococcus</i> sp. Lancefield's group B (3250)	+	21	0.4
<i>Streptococcus pneumoniae</i> (3399)	+	23	0.4
<i>Streptococcus pyogenes</i> (2723)	+	25	0.2
<i>Vibrio parahaemolyticus</i> (820)	–	14	1.2
<i>Yersinia enterocolitica</i> (2603)	–	20	0.4
² <i>Bacterioides fragilis</i>	–	25	0.2
² <i>Clostridium perfringens</i>	+	23	0.4
Insensitive Organisms (NZRM#)	Gram -/+	¹ Zone	Est. MIC
<i>Aeromonas hydrophila</i> (3205)	–	8	≥ 8.0
<i>Candida albicans</i> (2228)	(yeast)	0	> 8.0
<i>Enterococcus faecalis</i> (2244)	+	8	≥ 8.0
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> (3407)	–	0	> 8.0
<i>Pseudomonas aeruginosa</i> (918)	–	0	> 8.0
<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i> (1010)	+	7	≥ 8.0

¹Six mm Φ disks containing 10 $\mu\text{g} \cdot \text{ml}^{-1}$ AGA were placed on standardized lawns of each strain on Mueller-Hinton Blood agar, or LB agar, incubated under appropriate conditions for each strain, and the diameter of the zone of inhibition caused by AGA was measured in mm. MIC estimations (Est.) are derived from disk diffusion data.

²Obligate anaerobes were assayed under anaerobic conditions. *C. perfringens* was tested by staff in the Dept. Microbiology, Wellington Medical School.

4.5. Preliminary investigations into the mode of action of AGA

4.5.1. AGA is bacteriocidal

Previously, the antibiotic produced by Eh1087 was reported to be bacteriocidal on the basis that no viable cells could be recovered from the zone of inhibition around a colony of Eh1087 on an Ea8862 lawn. During this investigation purified AGA was found to have the same property. Furthermore, cultures of Ea8862 grown to exponential phase or to stationary phase were found to be killed by AGA, indicating that the killing activity is independent of growth-phase. Even sub-MIC doses of AGA killed Ea8862 cells within 30 min (Fig. 4.7). When the dose was greater than the MIC, most cells were killed within five min (data not shown). Interestingly, when AGA-killed and untreated Ea8862 cells were fixed, stained with crystal violet, and viewed with a light microscope at 1000 x magnification, no morphological differences were observed.

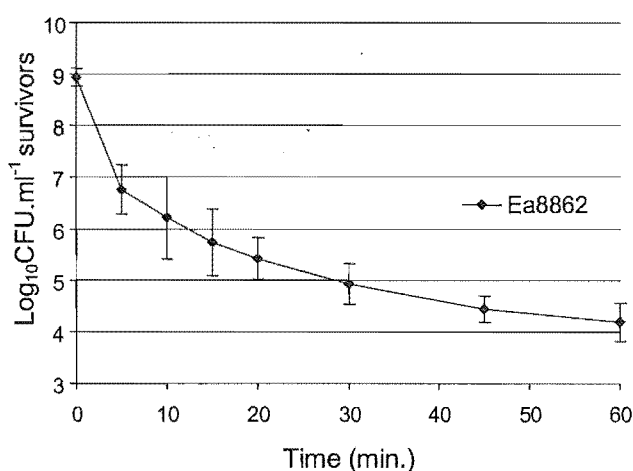


Figure 4.7. Survival of Ea8862 treated with Eh1087 antibiotic

Ea8862 cells from an overnight culture were suspended to ca. 1×10^9 CFU.ml⁻¹ in minimal media salts and treated with 0.08 μ g.ml⁻¹ AGA (ca. 50 % MIC). Samples were taken at the time intervals indicated, immediately rinsed twice with ice-cold minimal medium and enumerated by serial dilution. Data points were measured in triplicate.

4.5.2. AGA induces an SOS response

Many stress stimuli, particularly those that cause DNA damage, induce a regulon of genes that are normally repressed by LexA but become derepressed by the co-protease activity of RecA that has been exposed to single-stranded DNA (the DNA-damage signal). Collectively, these genes are known as the SOS regulon, and the induction of these genes can be assessed by

measuring the expression of the appropriate in-frame *lacZ* fusions (Walker, 1984). For instance, isogenic *E. coli* strains DE880 (*recA*⁺) and DE1491 (*recA*⁻) carry a fusion of *lacZ* to *sulA* which is induced as part of the SOS regulon to inhibit cell division. Under SOS conditions the activity of SulA is thought to stop the formation of DNA-replication induced DNA lesions and provide time for cell repair mechanisms to fix DNA damage. Measurement of β -galactosidase activity in DE880 is indicative of SOS induction, whereas DE1491 cannot induce an SOS response unless *recA* is provided in trans (Ennis et al., 1989). To test whether AGA induced *sulA*, and by inference an SOS response, these strains were treated with varying concentrations of AGA. 1.0 $\mu\text{g}.\text{ml}^{-1}$ AGA (ca. the MIC of AGA for this strain) induced the expression of *sulA* in DE880 to a similar level as 0.5 $\mu\text{g}.\text{ml}^{-1}$ mitomycin C, a well known inducer of the SOS response (Wei et al., 2001). The expression of *sulA* in response to AGA exposure by DE880 but not DE1491 demonstrates that AGA causes *recA*-dependent *sulA* induction (Fig. 4.8A). The *recA*-dependent induction was confirmed when pKS*recA* (a clone of the Eh1087 *recA* gene) was introduced to DE1491 and transformants were treated with AGA. In this strain *sulA* expression was induced to a similar level as AGA-treated DE880 cells. Cells treated with GA did not induce *sulA* expression, thus the SOS response correlates with antibiotic activity (data not shown). Also of significance, the induction of *sulA* by AGA was found to occur in a concentration dependent manner, and provides a very sensitive and accurate measure of antibiotic activity for AGA concentration well below the MIC for these strains (Fig. 4.8). *sulA* is the only member of the SOS regulon that has been assessed for induction by AGA, however because this induction is *recA*-dependent it is likely to represent an SOS response and will therefore be referred to as such from this point.

EhpR provides a cell with protection from AGA. If the SOS response in *E. coli* exposed to AGA corresponds to the antibiotic activity of AGA then it was hypothesized that the provision of antibiotic resistance by EhpR would reduce or eliminate the SOS-response of antibiotic treated *E. coli* cells. This hypothesis was supported by the finding that the SOS response was not observed in DE880 that had been transformed to AGA-resistance with pBB7::*ehpA* (Fig. 4.8B). The elimination of the SOS response by EhpR indicates that the EhpR-mediated AGA-resistance mechanism inhibits antibiotic activity before an SOS response is induced, and must therefore prevent even sub lethal amounts of AGA from acting on a target cell.

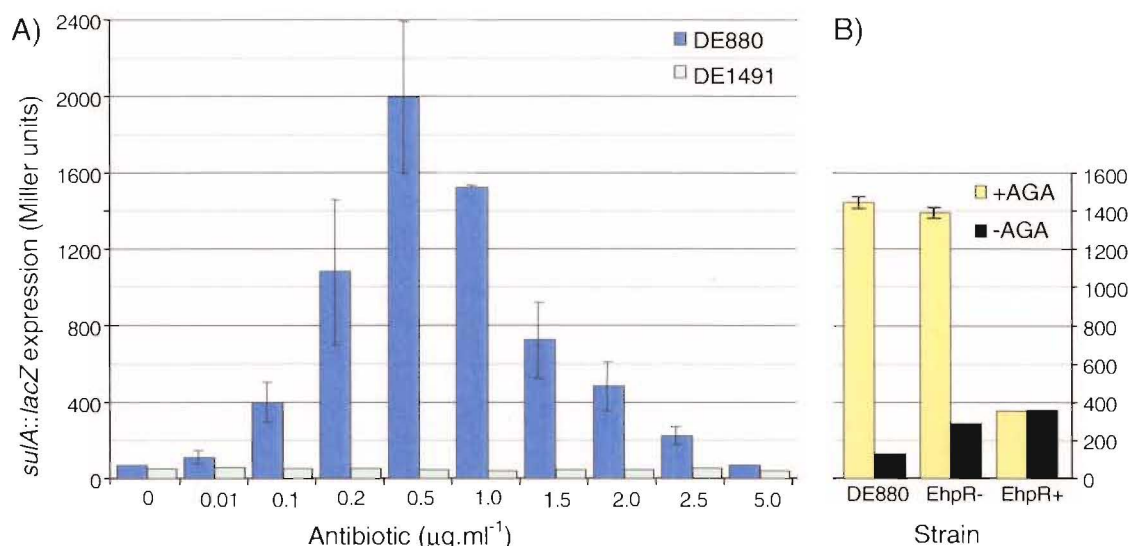


Figure 4.8. SOS induction in *E. coli* in response to AGA

A) AGA causes RecA-dependent induction of *sulA*. *E. coli* DE880 (*recA*⁺) and DE1491 (*recA*⁻) cells grown to an OD₆₀₀ of 0.5 in minimal media were treated with various amounts of crude Eh1087 antibiotic extract for 1 hour at 37 °C, in a water bath shaking at 250 rpm, and β -galactosidase activity was measured as described previously (Miller, 1972).

B) *sulA* induction is prevented by EhpR. *E. coli* DE880 and derivatives harbouring pBB7::*ehpR* (EhpR-) or pBB7::*ehpA* (EhpR+) were grown in LB to an OD₆₀₀ of 0.5, and treated with 1 $\mu\text{g.ml}^{-1}$ AGA. Background β -galactosidase activity conferred by pBB7::*ehpR* is 100-200 units, and for pBB7::*ehpA* is 200-300 units. MIC data for these strains: DE880 and DE880 (pBB7::*ehpR*), 0.4 $\mu\text{g.ml}^{-1}$; DE880 (pBB7::*ehpA*), > 16 $\mu\text{g.ml}^{-1}$; DE1491, 0.8 $\mu\text{g.ml}^{-1}$.

4.5.3. AGA is an indirect mutagen

A common stress known to induce the SOS response is DNA damage. Since AGA induced an SOS response and many phenazines are mutagenic (Kato et al., 1994; Martinez et al., 2000; Watanabe et al., 1989; Watanabe et al., 1989), the mutagenicity of AGA was assessed. Preliminary investigations into *E. coli* strains dosed with varying concentrations of AGA suggested that resistance to Rf^R and Nal^R, but not Sm^R, arose at significantly higher rates than in untreated cells (data not shown). When isogenic *recA*⁺ and *recA*⁻ strains were dosed sufficiently to reduce the population size by an order of magnitude and then allowed to fully recover, Rf^R and Nal^R mutants arose independently of *recA*, thereby excluding the error-prone repair mechanism that forms part of the SOS response from involvement in the mutagenesis. However, Rf^R and Nal^R mutants could have arisen because of DNA base alterations caused by

AGA, or because such mutants may already have existed in the population and possessed some advantage that enabled them to out compete their non-resistant peers when exposed to AGA.

To confirm whether or not AGA has in vivo mutagenic activity, a collection of six *E. coli lacZ* reversion tester strains (WP3101-WP3106) were obtained from Dr Toshihiro Ohta (School of Life Science, Tokyo University of Pharmacy and Life Science, Japan). These six strains are derived from *E. coli* B/r WP2 [F⁻, *uvrA155*, *trpE65*, *malB15*, *lon-11*, *sulA11*] and possess a F' plasmid carrying one of six possible unique *lacZ* mutations at codon Glu-461 resulting in a *lacZ*⁻ phenotype (Table 4.1). Reversion of these mutations is required for a *lacZ*⁺ phenotype, which can be assayed by plating bacteria on minimal media with lactose as the sole carbon source. If the frequency of reversion increases after exposure to a compound, then that compound is deemed mutagenic and the nature of the reversion indicates the type of mutation caused by the mutagen. It is worth noting that these strains are *uvrA*⁻ and therefore lack the ability to activate the excision repair pathway that would otherwise repair many mutations. The addition of plasmid pKM101 carrying *mucAB* to these six strains resulted in the creation of strains WP3101P-WP3106P. *mucA* and *mucB* are probable homologs of *umuD* and *umuC* and increase the rate of error prone repair under conditions in which DNA is damaged. Therefore different mutation rates between strains with and without *mucAB* indicates that the error-prone repair pathway is involved in the mutagenic process, and vice versa. The twelve *E. coli lacZ* reversion strains were exposed to 0.3 µg per plate AGA or 20 µg per plate sodium azide (as a positive control) at 37 °C for 20 min as described by Ohta (1998), plated on defined minimal media with lactose as the sole carbon source, and incubated at 37 °C for 48 h. After incubation, colonies that could be clearly seen by eye were counted (Table 4.5). In this initial experiment, AGA increased the rate of transition from G:C→A:T by approximately 10 times background, but caused no other base pair changes above background level. Since the frequency of revertants was lower in cells containing *mucAB*, it is apparent that the mutagenic process involving AGA does not depend on error-prone repair. This experiment was repeated using concentrations of AGA and AP3 from 0.05 µg to 32 µg per plate (data not shown). The mutagenic effect of AGA was concentration dependent, with the greatest number of revertants occurring at between 0.25 and 1.0 µg AGA per plate. Most bacteria were killed by ≥4 µg AGA per plate (0.16 µg.ml⁻¹ media). It was also noted that the number of revertants varied greatly from experiment to experiment, and that on most occasions the greatest reversion frequency after exposure to AGA was only 4-5 times greater than that of the untreated control. No increase in reversion was noted for bacteria cultured on plates supplemented with AP3 at various concentrations, suggesting that the antibiotic activity of AGA is related to mutagenicity.

Table 4.6. Reversion of *lacZ* point-mutations in *E. coli* strains exposed to AGA

Strain	Reversion	Control	NaN ₃	AGA
WP3101	A:T→C:G	0	1	1
WP3102	G:C→A:T	6	113	73
WP3103	G:C→C:G	0	0	0
WP3104	G:C→T:A	3	4	1
WP3105	A:T→T:A	1	4	7
WP3106	A:T→G:C	0	1	0
WP3101P	A:T→C:G	2	1	3
WP3102P	G:C→A:T	4	102	36
WP3103P	G:C→C:G	0	0	0
WP3104P	G:C→T:A	3	10	4
WP3105P	A:T→T:A	0	3	7
WP3106P	A:T→G:C	5	1	6

Data from one representative experiment is reported, in which the reversion of *E. coli* tester strains to *lacZ*⁺ in the presence of AGA of NaN₃ was assayed. 20 µg per plate NaN₃ was used as a positive control for G:C→A:T transitions (Ohta *et al*, 1998). AGA was used at 0.3 µg per plate. Strains WP3101-WP3106 grew more quickly and formed colonies with less size variation than WP3101P-WP3106P after exposure to AGA.

Does AGA act directly on DNA or does it require cellular components or physiological processes for mutagenic activity? To crudely test this question, purified DNA of the plasmid pUC19 was exposed to AGA for 24 and 72 hours, the pUC19 DNA repurified and assessed for ability to complement the α-fragment of *lacZ* in *E. coli* DH5α. Most mutations to the β-fragment of *lacZ* carried on pUC19 should inactivate β-galactosidase activity. AGA did not increase the proportion of *lacZ* mutations above background, whereas exposure to hydroxylamine did (Table 4.1). Therefore AGA does not cause mutation of DNA in the *in vitro* conditions tested, suggesting that AGA does not act directly on DNA, at least not in the absence of certain cellular component(s). Evidence presented in the next section suggests that the AGA may need an electron acceptor/donor such as NADP, an oxidoreductase, to effect its activity, however this has not been assessed *in vitro* against a target DNA molecule such as pUC19.

Table 4.7. Mutagenesis of pUC19 by AGA *in vitro*

Treatment	Colonies per plate (DH5 α <i>recA</i> +)	
	blue (wild type)	white (mutant)
Control (buffer only)	ca. 4000	2
Antibiotic	ca. 4000	2
Hydroxylamine	ca. 1000	70

DNA was exposed to AGA or hydroxylamine for 24 hours (similar results were obtained after 72 hours).

4.5.4. AGA has redox activity

The antibiotic activity of phenazine compounds is thought to occur by one of two common mechanisms, either by binding DNA and inhibiting various DNA-dependent processes, or by the generation of damaging oxidative compounds (Baron and Rowe, 1981; Behki and Lesley, 1972; Hassan and Fridovich, 1980; Hollstein and Van Gemert, 1971; Hori et al., 1978). AGA was shown to be weakly mutagenic against *E. coli*, causing G:C→A:T transitions, possibly via the oxidative deamination of cytosine to uracil (and the subsequent pairing with adenine rather than guanine). On this basis, an investigation into the ability of Eh1087 to cause free radical or oxidative damage was carried out in association with the Free Radical Research Group at the Christchurch School of Medicine, under the guidance of Dr Christine Winterbourne. Redox activity was assessed using two assays; an ‘in vivo’ assay in which the oxidation of haemoglobin released from lysed red blood cells was measured (all red cell components were present), and an ‘in vitro’ assay in which the reduction of commercially prepared cytochrome C was measured.

Oxyhemoglobin oxidizes to methemoglobin, and in some circumstances to other compounds such as hemichrome, or a methemoglobin::H₂O₂ complex known as ferryl-hemoglobin. The relative concentrations of these compounds can be derived from spectral changes as described by Winterbourne (1985). To assess whether or not AGA could oxidize hemoglobin, red blood cell lysate was treated with AGA and the change in absorbance spectra over time was measured. It is worth noting that high concentrations of AGA (32 or 64 $\mu\text{g}.\text{ml}^{-1}$) were required to observe clear hemoglobin oxidation, relative to concentrations sufficient to kill many bacterial spp. (0.2 – 4.0 $\mu\text{g}.\text{ml}^{-1}$). From the spectra data the change in oxidation state of oxyhemoglobin was determined (Fig. 4.10). The treatment of red blood cell lysate with AGA or AP3 resulted in a decrease in the oxyhemoglobin fraction over time; concurrently an increase in the oxidized oxyhemoglobin derivative methemoglobin is observed and in the absence of catalase activity it

appears that the ferryl-hemoglobin (methemoglobin: H_2O_2) complex also formed. Because the rate of these changes increases when the concentration of AGA or AP3 is increased, and since no hemoglobin oxidation was observed in reactions lacking AGA or AP3, it appears that these compounds are acting as oxidants. Originally the data was treated with the Winterbourne equations (see materials and methods) which indicated that no hemichrome was formed (data not shown), but the oxidized hemoglobin products could not account for the decrease in oxyhemoglobin concentration. However, the Van den Berg modified equations using the correct figures of Whitburn (for H_2O_2 adducts) suggested that ferryl-hemoglobin was forming rather than hemichrome. Hemichrome formed when the red blood cell lysate was treated with the oxidant acetylphenylhydrazine, confirming that the lack of hemichrome formation is a specific feature of the hemoglobin oxidation involving AGA (data not shown). Two lines of evidence suggest that the oxidation of hemoglobin by AGA and AP3 involves the generation of reactive H_2O_2 ; firstly, the formation of ferryl-hemoglobin, and secondly, the finding that in the presence of azide to inactivate catalase and maintain H_2O_2 , oxyhemoglobin was oxidized at a higher rate and methemoglobin was not detected (Fig. 4.9). Thus, the oxidation of hemoglobin by AGA appears to cause the formation of a methemoglobin- H_2O_2 complex. It is also apparent that AP3 oxidizes oxyhemoglobin at a lower rate than AGA by a small but consistent margin, for reasons that remain to be determined.

Once it was established that AGA and AP3 caused haemoglobin to become oxidized to methemoglobin in the presence of catalase and probably to ferryl-hemoglobin in the absence of catalase (Fig. 4.9), the next step was to determine if AGA and AP3 could reduce cytochrome C in the absence of the cellular components present in red cell lysate. The rate of cytochrome C reduction was measured as the change in A_{550} over time in reaction mixtures containing one or more of the following; cytochrome C, varying concentrations of AGA and AP3, the electron donor NADPH (or NADH), and ferredoxin-NADP⁺ reductase to recycle electron donors. The results of this assay are presented in Table 4.8. Reactions 1-6 (Table 4.8) demonstrate the relationship between AGA or AP3 concentration and the rate of cytochrome C reduction. Reactions involving equivalent concentrations of AGA and AP3 proceed at a very similar rate. As the concentration of either AGA or AP3 doubles, the rate of cytochrome C reduction almost doubles, although when the concentration of AGA or AP3 is $32\text{ }\mu\text{g.ml}^{-1}$ other factors appear to limit the reaction rate. Consequently, it can be concluded that, at least for $8\text{ }\mu\text{g.ml}^{-1}$ and $16\text{ }\mu\text{g.ml}^{-1}$, the reaction rate is directly proportional to the concentration of AGA and AP3, which are therefore involved in the reduction of cytochrome C. This conclusion is supported by the finding that the reaction does not proceed in the absence of AGA or AP3.

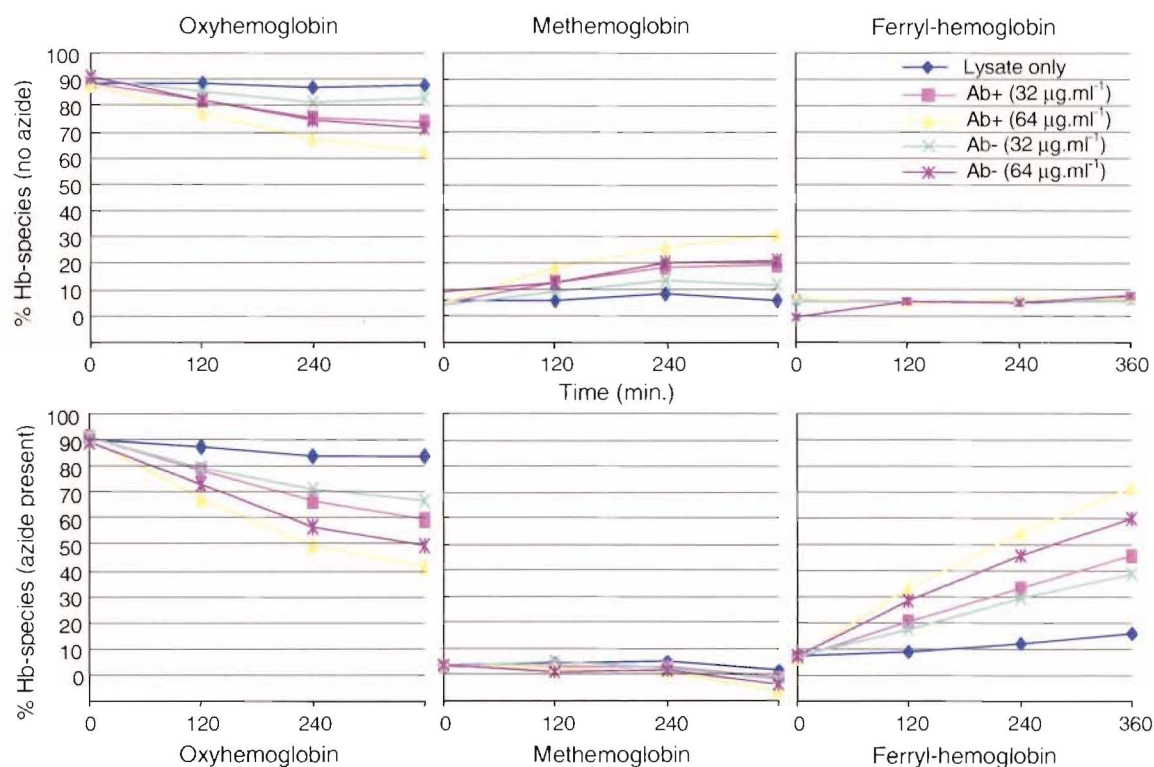


Figure 4.9. Oxidation of hemoglobin by AGA and AP3.

The concentrations of oxyhemoglobin, methemoglobin, and ferryl-hemoglobin (assumed) were derived from spectral measurements of red cell lysate exposed to 32 µg.ml⁻¹ and 64 µg.ml⁻¹ AGA or AP3 at 37 °C. Samples were taken at 2 h intervals for 6 h. Reactions were carried out with azide absent (top three graphs) or in the presence of azide (bottom three graphs). Azide inactivates catalase and inhibits the degradation of H₂O₂, hence H₂O₂ is absent in the assay reported in the top three graphs and present in the assay reported in the bottom three graphs.

The effect of limiting NADPH or ferredoxin-NADP⁺ reductase on the rate of cytochrome C reduction is demonstrated by reactions 7 – 13. Lowering NADPH concentration by 50 % decreased the rate of cytochrome C reduction by ca. 35 % for reactions containing either AGA or AP3, indicating that the concentration of NADPH has a strong influence on the reaction dynamics (reactions 7 and 8). In comparison, the presence of 30 µg.ml⁻¹ superoxide dismutase (to remove superoxide) or 35 µg.ml⁻¹ catalase only decrease the reaction rate by ca. 12 % or ca. 8 %, respectively, indicating that superoxide and H₂O₂ have only a minor role in the reduction of

cytochrome C mediated by AGA or AP3 (data not shown). It has been proposed that superoxide acts in the auto-oxidation of fully reduced phenazine derivatives, restoring their electron-accepting potential (Davis and Thornalley, 1983).

Table 4.8. Cytochrome C reduction by AGA and AP3

Reaction	¹ AGA ($\mu\text{g}.\text{ml}^{-1}$)	¹ AP3 ($\mu\text{g}.\text{ml}^{-1}$)	² NADPH (μM)	³ Reductase (units)	⁴ Slope	⁵ Rate ($\mu\text{M}.\text{min}^{-1}$)
1	8	-	100	0.02	0.0571	2.71
2	16	-	100	0.02	0.0987	4.68
3	32	-	100	0.02	0.1481	7.02
4	-	8	100	0.02	0.0544	2.58
5	-	16	100	0.02	0.0969	4.59
6	-	32	100	0.02	0.1464	6.94
7	8	-	50	0.02	0.0370	1.75
8	-	8	50	0.02	0.0364	1.73
9	8	-	100	0.01	0.0275	1.30
10	16	-	100	0.00	0.0315	1.49
11	32	-	100	0.00	0.0529	2.51
12	-	16	100	0.00	0.0052	0.25
13	-	32	100	0.00	0.0088	0.42

Reactions showing cytochrome C reduction only are reported; no reduction was observed if either NADPH or AGA / AP3 were absent. The concentration of reduced cytochrome C at the end point of each reaction was calculated to be 28 mM ($A_{550} = 0.6$).

- 1 AGA and AP3 were extracted from cultures of Eh1087 and EhehpO respectively, equilibrated for A_{364} , and standardized by measuring the antibiotic activity of the AGA sample relative to purified AGA.
- 2 NADH produced similar results when substituted as the electron donor.
- 3 One unit of ferredoxin-NADP⁺ reductase reduces 1.0 μM NADP per min at pH 7.6, 25 C.
- 4 The plot of A_{550} change over time in min (reactions proceeded for up to 12 min).
- 5 The rate of cytochrome C reduction. In these conditions a positive control, 200 μM menadione (a redox-cycling agent), reduced cytochrome C at 20.9 $\mu\text{M}.\text{min}^{-1}$.

Ferredoxin-NADP⁺ reductase is a limiting factor in the reduction of cytochrome C since lowering the concentration of this enzyme by 50 % decreases the reduction rate by ca. 50 % (reactions 1 and 9). Removing the enzyme altogether reveals an important difference between AGA and AP3 (reactions 10-13). The rate of reactions without reductase and in the presence of AGA, is decreased by ca. 65 % initially and the reaction rate slows rapidly after 1 min (data not shown). In contrast, reactions containing AP3 and no reductase proceed at ca. 5 % of the rate of reactions containing reductase. Because the rate at which reductase replenishes NADPH is too low to affect the reaction rate (one unit will reduce 1.0 μ mole NADP per min), the reductase probably mediates redox reactions between cytochrome C and AGA or AP3 (Fig 4.11). The most likely scenario is that NADPH-reduced ferredoxin-NADP⁺ reductase reduces AGA and AP3, which are subsequently able to reduce cytochrome C. Additionally, it is likely that the redox potential of AGA is sufficiently high to oxidize NADPH and reduce cytochrome C in the absence of reductase, at least for a short period, whereas AP3 does not have the appropriate redox potential for this reaction. The end product of AGA / AP3 oxidation is likely to be the semiquinone free radical illustrated in Figure 4.10, a molecule that can react with either two-electron or one-electron donor-acceptor pairs in a similar manner to the flavins involved in pyruvate oxidation.

4.5.5. Catalase does not protect bacteria against the antibiotic activity of AGA

The presence of catalase prevented the formation of methemoglobin::H₂O₂ complexes during the oxidation of hemoglobin by AGA, but had little effect on the rate of cytochrome C reduction. To determine whether or not H₂O₂ could be involved in the antibiotic activity of AGA, the potential for a potent catalase from *S. entomophila* (Giddens, 1995) to provide bacteria with protection against AGA was assessed using the disk diffusion assay (Table 4.7). *S. entomophila* is intrinsically resistant to AGA (MIC and MLC, 16.0 μ g.ml⁻¹). A catalase-minus mutant of *S. entomophila* (BC4BCR) is as resistant to AGA as the catalase-producing parent strain indicating that catalase is not involved in the high inherent AGA-resistance of *S. entomophila*. Furthermore, *E. coli* HB101 harboring a plasmid containing the catalase gene (pSEC1) was equally as sensitive as HB101 harbouring either no plasmid or a plasmid with an inactive catalase gene (pSEC1-1), demonstrating that the *S. entomophila* catalase does not provide a heterologous host with any level of AGA-resistance. This data suggests that the antibiotic activity of AGA is not dependent on the presence of H₂O₂.

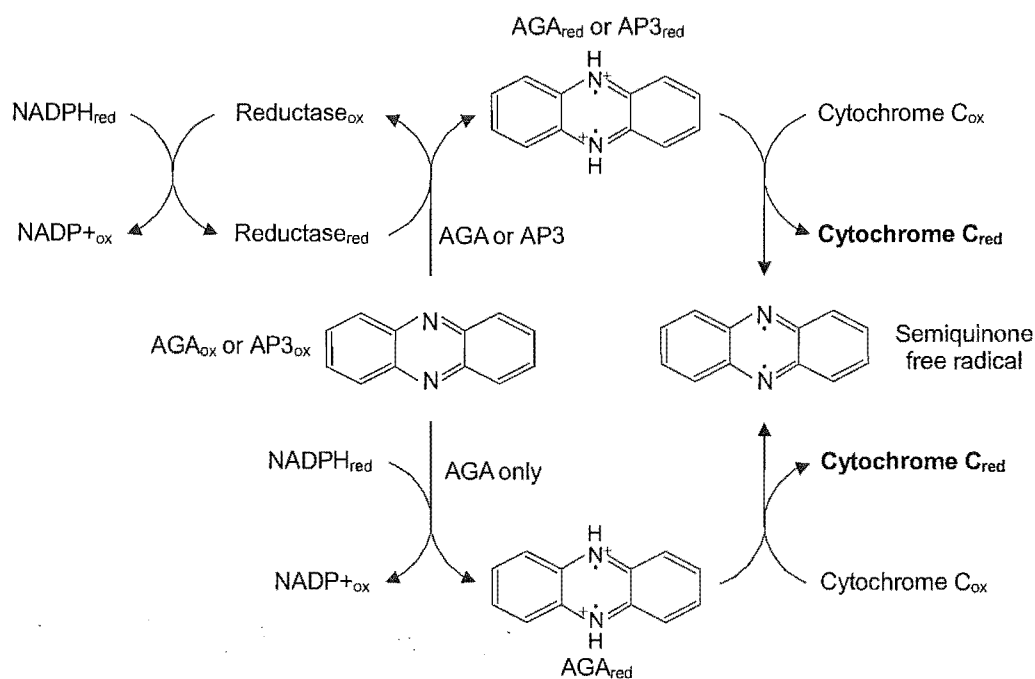


Figure 4.10. Proposed redox reactions of AGA and AP3 during cytochrome C reduction

AGA or AP3 can reduce cytochrome C when ferredoxin-NADP+ reductase is present (top section), whereas only AGA can mediate this reaction in the absence of the enzyme (bottom section). All reactive AGA and AP3 derivatives are currently theoretical, and all reactions are in equilibrium.

Table 4.9. Sensitivity of bacteria to AGA in the presence of catalase

Strain	Catalase	MIC	1 $\mu\text{g}.\text{ml}^{-1}$	10 $\mu\text{g}.\text{ml}^{-1}$
<i>S. entomophila</i> BC4B	+	16	0	7
<i>S. entomophila</i> BC4BCR	-	16	0	7
<i>E. coli</i> HB101	-	0.4	13	22
<i>E. coli</i> HB101 [pSEC1]	+	0.4	13	22
<i>E. coli</i> HB101 [pSEC1-1]	-	0.4	13	22

MIC data was measured for BC4B and estimated for all other strains. Six mm disks containing 1 or 10 $\mu\text{g}.\text{ml}^{-1}$ AGA were placed on standardized lawns of each strain, which were incubated at 30 °C (*S. entomophila* strains) or 37 °C (*E. coli* strains). Clearing zones were measured after 18 hr, and catalase activity was confirmed by placing a drop of 100 v/v H₂O₂ on the lawn surface and observing the vigorous release of O₂.

Discussion

Eh1087 produces antibiotic during periods of growth in minimal and rich media, with maximum production during entry to stationary phase as illustrated in Figures 4.1 and 4.2. Similarly the production of AGA by Eh1087 in planta also correlated with periods of growth (Chapter 2). This extends the previous observations of Kearns and Hale (1996) who detected antibiotic in the supernatants of cultures entering stationary phase. In contrast, many microbes do not produce antibiotics in medium supporting rapid growth (Malik, 1979). It has been noted that phenazine production in *P. phenazinium* reduces the maximal population size relative to a non-producing mutant derivative, SP2 (Messenger and Turner, 1981). Whereas for SP2 the non-producing mutant lost viability more quickly than the phenazine producing strain during stationary phase, the opposite is observed for Eh1087. The population decrease of Eh1087 relative to Eh Δ AGA could be caused by AGA toxicity, or the metabolic burden of producing AGA. Either way, once the concentration of AGA drops sufficiently, Eh1087 multiplies again and produces antibiotic. This experiment did not demonstrate the point during growth that Eh1087 produces the most antibiotic per cell, but it did identify the optimal time to harvest Eh1087 cultures and maximize the antibiotic yield for subsequent investigations.

Antibiotic production by Eh1087 initiates during early-log phase in defined minimal media and in mid-log phase in rich media (Fig. 4.2). In contrast, phenazines are usually synthesized after exponential phase, and this has been associated with an increase in aromatic amino acid synthesis (Byng and Turner, 1975; Turner and Messenger, 1986). It also appears that some constituent(s) of rich media causes inhibition of antibiotic production by Eh1087 during early exponential phase growth. A comparable situation occurs in *E. coli* harbouring the AGA gene cluster, although in these strains the effect is far more severe, and AGA production is often not detected at all in rich media. These observations agree with *ehp* gene expression patterns in *E. coli* and Eh1087 (section 3.7). It is therefore probable that rich media suppression does operate in Eh1087, although to a lesser degree than in *E. coli*, and that Eh1087 possesses a mechanism to overcome rich media suppression that is additional to the AGA gene cluster.

The partial suppression of antibiotic production by Eh1087 grown in nutrient-rich media may be caused by aromatic amino acid feedback inhibition of the shikimic acid pathway thereby reducing synthesis of the common phenazine precursor, chorismic acid (Carson and Jensen, 1974; Longely et al., 1972). This hypothesis is supported by the fact that Eh1087 produces less antibiotic in the presence of excess tyrosine (data not shown). Such feedback inhibition may act

on the first dedicated step in the shikimic acid pathway, catalyzed by 3-deoxy-D-arabinoheptulosonate-7-phosphate (DAHP) synthase. DAHP synthase repression of aromatic amino acids in phenazine producing *Pseudomonads* has been demonstrated by Jensen et al. (1967) and Levitch (1970) who found that of the aromatic amino acids only tyrosine was inhibitory in 16 different phenazine-producing strains, and Byng and Turner (1975) who found that only phenylalanine significantly inhibited this enzyme in *P. phenazinium*. Conversely, the PCA operons now characterized in a number of *Pseudomonads* (see Fig. 3.8) have a putative DAHP synthase isozyme, PhzB/PhzE, that may not be susceptible to feedback inhibition by aromatic amino acids, although this prediction has yet to be tested empirically. The lack of a potential homologue of DAHP synthase in the AGA gene cluster could explain why tyrosine inhibits AGA production in Eh1087.

The chemical structure of the antibiotic produced by Eh1087

The antibiotic produced by Eh1087 was extracted from culture supernatant to chloroform in much the same way as phenazine antibiotics are initially purified from other organisms (Byng and Turner, 1976; Chang and Blackwood, 1969; Gerber, 1969; Imamura et al., 1997). Extraction procedures are usually based on a protocol developed by Chang and Blackwood (1969) in which the culture supernatant is acidified prior to chloroform extraction. This process is suitable for the extraction of most phenazines, however the antibiotic produced by Eh1087 does not partition to chloroform from acidified culture supernatant (Fig. 4.3). An acid-extraction step was found to be useful however, as many other compounds that co-partition from Eh1087 culture supernatant to chloroform are insoluble at low pH, conditions in which the Eh1087 antibiotic is soluble and loses activity at a slow rate. This information led to the development of a simple antibiotic purification process as outlined in Section 4.2. In fact, antibiotic purified by this method was usually suitable for NMR analysis. Further purification of the Eh1087 antibiotic by gel filtration chromatography yielded antibiotic with no detectable contaminants that was suitable for structural elucidation and quantification.

The antibiotic produced by Eh1087 was determined to be the phenazine compound 1-carboxy-6-D-alanyl-9-methoxy-phenazine (D-alanylgriseoluteic acid (AGA), Fig. 4.6). An extensive screen of the literature indicates that phenazine compounds have not been isolated from the *Erwinia* (*Pantoea*) genus before, and that AGA has only been isolated on one previous occasion, from a species of marine *Vibrio*, SANK 73794 (Sato et al., 1995). Therefore, to the authors knowledge this is the first report describing the isolation of the phenazine compound AGA from a terrestrial organism. Similar compounds, containing 3-hydroxyvalyl (HVGA),

valyl (VGA), or glycyl (GGA) esters in the same position as the D-alanyl ester in AGA, have recently been described (Imamura et al., 1997; Sato et al., 1995; Singh et al., 1997). These antibiotics were given the common name Pelagiomycin A, B, or C respectively, due to their isolation from another marine bacterium, *Pelagibacter variabilis* (Imamura et al., 1997). The pelagiomycins and all analogues containing an amino acid ester-bound to the 6-hydroxymethyl group of griseoluteic acid (see Fig. 4.6), are protected by Japanese patent Toku-Kai-Hei 9-48764 (18 February 1997) held by Nobutaka Imamura, Hiroshi Sano, and Takahide Takadera (see Imamura et al., 1997), of the Marine Biotechnology Institute, Shimizu Laboratories, Japan. This patent specifically excludes HVGA, presumably because it was described in the literature near to the time that the patent was lodged (Singh et al., 1997). Curiously, the patent does not specifically exclude AGA, however since this compound was described in the literature prior to patent application (Sato et al., 1995), it is unlikely that AGA is subject to patent protection.

AGA is unstable in water or alcohol and degrades to a red, non-polar compound, determined to be 1-carboxy-6-hydroxymethyl-9-methoxy-phenazine (griseoluteic acid, GA). GA is therefore the first known phenazine compound to be isolated from an *Erwinia* (*Pantoea*) species, and it arises from the loss of the D-alanine residue from AGA (Fig. 4.6). GA was also isolated from bacteria producing griseoluteins and pelagiomycins, suggesting that this reaction is common to other GA-derived compounds (Imamura et al., 1997; Sato et al., 1995; Singh et al., 1997). GA was originally isolated from *Streptomyces griseoluteus* as a breakdown product of griseoluteins A and B (Nakamura, 1958). Griseolutein A has a glycolic acid moiety at the 6-hydroxymethyl group of GA, which is thought to be hydrogenated to create griseolutein B (Yagishita, 1960). Both compounds are easily hydrolyzed to reform GA. Because GA has very low antibacterial activity relative to the griseoluteins, the modifications at the 6-hydroxymethyl group were considered important for antibiotic activity (Yagishita, 1960). This prediction has been strengthened by similar findings with more recently discovered GA derivatives modified at the same position, such as AGA (this study; Sato et al., 1995), HVGA (Imamura et al., 1997; Singh et al., 1997), and glycylgriseoluteic acid (Sato et al., 1995). In particular, to account for the higher antibiotic activity of HVGA relative to griseoluteic acid, both isolated from culture LL-141352, Singh et al. (1997) proposed that the amino acid residue facilitated the transport of the antibiotic across the cytoplasmic membrane. The amino acid group may enable these GA-derivatives to be taken up by transporters similar to the non-specific di- and tripeptide transporters that are present in bacteria and higher organisms. For instance, PEPT1 is the model peptide transporter found in the mammalian intestine that has affinity for compounds with amino-terminal and carboxyl-terminal groups separated by 500-650 pm or the approximate equivalent of four CH₂ units (Doring et al., 1998). Doring et al (1998) proposed that one

charged group is sufficient for anchoring and that a second oppositely charged group is required for transport by PEPT1. Phenazines may be too long to be taken up by this transporter, however the same principle could apply to other non-specific transporters and offer a mechanism to differentiate between GA, which has only a single charged carboxyl group, and the amino acid substituted derivatives. Furthermore, the ubiquity of these transporters would provide an explanation for the broad spectrum of organisms that are susceptible to the GA-derived antibiotics. These propositions could be tested by assessing the sensitivity of different transport mutants to the GA-derivative antibiotics that have been recently described.

Properties of the AGA intermediates provide clues about the AGA biosynthetic pathway

The coloured AGA precursors could be differentiated from each other and from AGA by their differential partitioning between chloroform and culture supernatants at acid or neutral pH. The first AGA phenazine precursor, AP1, only extracts to chloroform from acidified supernatant medium. The second precursor AP2, does not extract to chloroform at neutral pH and precipitates out of solution at low pH, while the third AGA precursor, AP3, is insoluble at low pH but does extract to chloroform at neutral pH. These data demonstrate the varied chemical properties of each of the AGA intermediates and can be used with other data such as colour, solubility, and absorbance maxima, to estimate the type of phenazine present at each stage of AGA synthesis even though the chemical structures of these intermediates are not yet known (Fig. 4.7). For instance, AP1 has similar properties to PCA, and AP3 has similar properties to GA. The minor additions to the phenazine nucleus predicted for AP1, and the more substantial modifications predicted for AP3 agree well with the proposed anabolic nature of the gene products responsible for the intervening modifications. The properties of AP2 were less well defined because this compound did not extract to chloroform for similar analyses.

Antibiotic activity and cytotoxicity of GA-derived antibiotics

The potency of AGA purified from a culture of Eh1087 against a range of Gram+ and Gram- organisms is shown in Table 4.4. Sato et al. (1995) reported that AGA purified by a different method from a different bacterium (*Vibrio* SANK 73794) had similar activity and inactivity against *S. aureus* and *P. aeruginosa*, respectively. They also showed that the MIC of AGA and GGA against a range of organisms were generally the same or varied only slightly. Imamura et al. (1997) found that HVGA was less active against *S. aureus* and more active against *P. aeruginosa*, but was inactive against the yeast *Candida albicans*, as observed for AGA in this study. In contrast, Singh et al (1997) reported that HVGA was only weakly active (MIC > 16 $\mu\text{g.ml}^{-1}$) against five unspecified Gram- isolates. Only AGA purified from Eh1087 has been

tested against anaerobic organisms, however it is likely that all GA-derived antibiotics have a similar mode of action, and therefore all would be similarly active against obligate anaerobes. Additionally, another phenazine antibiotic, pyocyanin, is active both aerobically and anaerobically (Baron and Rowe, 1981). In summary, all GA-derived antibiotics that have been reported possess a range of antibiotic activities against bacterial isolates, and this activity varies with respect to the susceptibility levels of these isolates. However, given the variation in reported potency for HVGA, these data should be considered tentative until all compounds are tested against the same isolates under the same conditions.

As with many other phenazine compounds the GA-derived antibiotics are cytotoxic. HVGA showed strong cytotoxic activity against HeLa, BALB373 and BALB3T3/H-ras cell lines ($0.04\text{--}0.2\text{ }\mu\text{g.ml}^{-1}$), and weak but unspecified activity against the murine P388 leukemia cell line in vivo (Imamura et al., 1997). Singh et al. (1997) tested HVGA against another five different tumor cell lines and found it to be less active than Imamura et al. (1997), reporting LD_{50} values of 0.12 to $0.7\text{ }\mu\text{g.ml}^{-1}$, but also noted that a normal endothelial cell line was less sensitive ($\text{LD}_{50} = 2.7\text{ }\mu\text{g.ml}^{-1}$). They also reported that HVGA concentrations up to 8 mg.kg^{-1} were inactive against a P388 murine leukemia model. In contrast, AGA purified from Eh1087 showed some activity in an in vitro test against the P388 cell line, ($\text{ID}_{50} = 0.43\text{ }\mu\text{g.ml}^{-1}$), and therefore further cytotoxic tests with AGA may be warranted (Y. Feng, pers. comm.). Unfortunately the therapeutic use of these compounds as anticancer agents may be limited by their relatively high acute toxicity, which is a measure of the dose sufficient to kill a mouse (LD_{100}). Singh et al. (1997) reported an LD_{100} value of 16 mg.kg^{-1} for HVGA, and Sato et al. (1995) reported the LD_{100} of GGA as less than 12.5 mg.kg^{-1} , but provided no data for AGA. The efficacy of these compounds in vivo is also questionable, as 8 mg.kg^{-1} HVGA did not protect mice against a lethal infection of *S. aureus*. Interestingly, most published data relates to HVGA, which is not protected by patent. Given that the potency of different GA-derived antibiotics varies between different bacterial isolates and between different cell lines, it is possible that other GA-derivatives such as AGA may be therapeutically useful.

Properties of AGA that are involved in antibiotic activity

The targets of most antibiotics produced by *E. herbicola* strains are potentially easy to detect because the antibiotic activity is inhibited by the presence of amino acids (El-Goorani and Beer, 1991; Ishimaru et al., 1988; Vanneste et al., 1992; Wodzinski et al., 1990; Wodzinski and Paulin, 1994). In contrast, the target of AGA is more difficult to determine as it is not inhibited by essential amino acids (Kearns and Hale, 1996), although this has not been reassessed with

purified AGA. Since little data regarding the mode of action of GA-derived antibiotics have been published, a number of general properties of AGA were investigated with the intention of determining the mode of action by which AGA kills a target organism.

Previously, it has been demonstrated that the phenazines PCA, PCN, pyocyanin, iodinin and myxin, among others, bind DNA (Hollstein and Van Gemert, 1971). This led to the proposition that at least part of the antibiotic activity of phenazine antibiotics was caused by DNA intercalation, which would result in the inhibition of RNA synthesis and presumably the inhibition of DNA replication. It was subsequently proposed that myxin inhibits DNA synthesis and causes breaks in single stranded DNA, eventually leading to general DNA degradation and cell death (Behki and Lesley, 1972). Behki and Lesley (1972) also found that myxin-induced DNA damage was incurred far more quickly if protein synthesis was inhibited by chloramphenicol, and proposed that the DNA damage is normally repaired by a repair 'enzyme' that is not expressed when protein translation is inhibited by chloramphenicol. The antibiotic griseolutein, which is structurally very similar to AGA, was shown to inhibit DNA replication, but not to affect the stability of synthesized DNA or the synthesis of RNA or protein molecules (Hori et al., 1978). The DNA repair 'enzyme' hypothesis of Behki and Lesley (1972) is still valid, as it is now well established that the inhibition of DNA replication at damage-induced lesions leaves gaps of ssDNA, which induce an SOS regulon comprised of numerous genes involved in repairing DNA and restoring cell health (Sassanfar and Roberts, 1990; Walker, 1984). DNA damage is quickly recognized by RecA, which associates with ssDNA and enhances the cleavage of the SOS regulon repressor, LexA. One member of the SOS regulon, Sula, interacts with FtsZ and prevents formation of the FtsZ ring to inhibit cell division and provide a damaged cell with time and resources to mend (Bi and Lutkenhaus, 1993). In support of the DNA intercalation model for phenazine antibiotic activity, AGA caused the *recA*-dependent induction of *sulA* expression in *E. coli* (Fig. 4.8). On this basis it is likely that AGA induces an SOS response, although the induction of further SOS-response genes would strengthen this claim. Also in support of this model was the finding that *recA* strains of *E. coli* are slightly more sensitive to AGA than isogenic *recA*⁺ strains. In comparison, the sensitivity of *recA*⁻ strains to agents that specifically act to damage DNA, such as 4-nitroquinoline-1-oxide or mitomycin C, is far greater than isogenic *recA*⁺ strains; the relative difference in sensitivity can be used to isolate *recA* genes based on the complementation of *recA* mutants for resistance to these compounds (Keener et al., 1984) (see also section 3). Thus, because *recA*⁺ strains are still relatively sensitive to AGA it is likely that DNA damage is not the primary antibiotic activity of this compound. Furthermore, because the SOS response is induced by generalized DNA damage it does not demonstrate the target of AGA or the processes by which AGA

generates DNA damage. Since any property of AGA that contributes to antibiotic activity warrants investigation and to obtain further information about the specific effect(s) of AGA on DNA, experiments were carried out to determine if AGA is mutagenic.

It has been demonstrated that many, but not all phenazines are mutagenic (Watanabe et al., 1989). In the course of general experimental work it was observed that AGA increased the frequency of rifampicin-resistant *E. coli*. Further testing demonstrated that the frequency of Rf^R consistently increased ca. 30 fold in the presence of AGA (data not shown). The mutagenic property of azide was discovered by the similarly fortuitous observation that it increased the frequency of penicillin- and streptomycin-resistant mutants in *Staphylococcus aureus* (recounted by Owais and Kleinhofs, 1988). Subsequently, AGA was shown to be weakly mutagenic, causing G:C→A:T transitions at 5-10 times background. The weak mutagenicity of AGA could be due in part to the position of phenazine ring substitutions at carbons 1, 6, and 9 (Fig. 4.6), because Watanabe et al (1989) found that only phenazines substituted at positions 2 and 7 are extremely mutagenic. AP3 was not mutagenic, indicating that without antibiotic activity these compounds are not mutagenic. However, the frequency of mutation caused by exposure to AGA was very low. Thus, both DNA damage and the associated mutagenesis are unlikely to make a major contribution to the mechanism by which AGA causes cell death.

The lack of direct DNA mutagenesis by AGA in vitro suggests that the process is indirect and requires one or more cell components. As discussed below, the mutagenic property of AGA may need to be 'activated' by the donation of an electron from a redox-active compound such as NAD(P)H. To compare the mutagenic activity of AGA in vitro with activity in vivo, *E. coli* cells harboring pUC19 could be subjected to antibiotic treatment and assessed for mutation. If pUC19 mutations do arise under in vivo conditions, it is anticipated that in vitro experimental conditions could be modified by the addition of cell lysate to determine whether cell metabolism or certain cell component(s) are required for mutagenesis resulting from AGA exposure. It may also be possible to show this under in vivo conditions by treating *E. coli* cells harboring pUC19 with bacteriostatic antibiotics that inhibit various cell functions, such as the expression of redox-cycling enzymes.

Does mutagenicity have any role in the antibiotic activity of AGA? Even if the mutagenic effect of AGA is an indirect result of the reactions that occur when a cell is exposed to AGA, it may influence the efficacy of AGA. An example of a defined G:C→A:T transition is the chemical modification of cytosine by nitrous acid. Nitrous acid converts cytosine to uracil by oxidative deamination and uracil then pairs with adenine rather than guanine during the

subsequent DNA replication round. Under 'normal' intracellular conditions when a catalyst like nitrous acid is not available, the oxidative deamination of cytosine occurs at a low background rate and the resulting uracil moieties are removed in *E. coli* and many other organisms by uracil-DNA-glycosylase (Duncan and Miller, 1980; Duncan et al., 1978; Duncan and Weiss, 1982). Thus, if the G:C→A:T transition induced by exposure to AGA is involved in antibiotic activity, an *E. coli* uracil-DNA-glycosylase mutant (*ung*-) should be hypersensitive to AGA due to an inability to remove uracil bases from its genome. Alternatively the induction of uracil DNA glycosylase activity by AGA could be assessed directly (O'Grady, 2000). Given that *recA* mutants are marginally more sensitive to AGA than *recA*⁺ isogenic strains, it could be useful to further test the relationship between mutagenesis and antibiotic activity by comparing the AGA-sensitivity of a range of DNA-damage repair mutants. The correlation between the oxidative deamination of uracil by nitrous acid and the weak G:C→A:T transition mutagenesis involving AGA was strengthened by the next series of experiments, which demonstrate that AGA is redox active.

Assuming that the mutagenic activity of AGA is insufficient for bacterial killing, what is the property of AGA that gives it antibiotic activity? The phenazine nucleus in AGA has two nitrogen that can be reduced (resulting in a semiquinone free radical, Figure 4.11) and could therefore potentially interfere in the electron transport chain (Crawford et al., 1986). It has been proposed that pyocyanin is directly reduced by NADH, producing a pyocyanin radical that diverts the normal electron flow in *E. coli* to generate toxic O₂ and H₂O₂ species (Hassan and Fridovich, 1980). Baron and Rowe (1981) demonstrated that pyocyanin was toxic in the absence of oxygen, and suggested that pyocyanin has sufficient redox potential to compete for electrons at several points in the electron transport chain itself. They also hypothesized that the antibiotic activity of pyocyanin could simply arise from the interruption of the electron transport chain. To compare the activity of AGA with these findings and to see if antibiotic activity correlated with redox activity, AGA and the non-toxic precursor AP3 were assessed for redox activity.

AGA and AP3 are redox active. Both were capable of mediating the oxidation of hemoglobin, however because this reaction required a high concentration of AGA and AP3 and took place over a number of hours it is unlikely to be a primary factor in antibiotic activity. Conversely, the reduction of cytochrome C in the presence of NADPH and ferredoxin NADH reductase was far more rapid, suggesting that the redox activity of AGA could be involved in antibiotic activity. NAD(P)H cannot directly reduce cytochrome C, but in reactions similar to those reported here, it was shown to reduce phenazine methosulphate (PMS), which can then reduce

cytochrome C rapidly (Picker and Fridovich, 1984). Superoxide was not detected in this reaction, presumably due to the direct activity of PMS on cytochrome C, and possibly also because phenazines such as PMS and 1-hydroxyphenazine can scavenge superoxide (Muller, 1995; Picker and Fridovich, 1984). The rate of cytochrome C reduction by AGA or AP3 is also not greatly affected by the removal of superoxide, probably for the same reasons. In contrast to PMS, when ferredoxin NADH reductase is absent the rate of cytochrome C reduction by AGA is considerably reduced and is almost non-existent for AP3. The reductase presumably mediates the redox activity between NADPH and AGA or AP3. Thus, the redox potentials of PMS, AGA, and AP3 are all different. Determination of the redox potentials of AGA and intermediate compounds would be interesting given that phenazines with more positive reduction potentials are reported as having the highest antibiotic potency (Crawford et al., 1986).

How is the redox activity of AGA involved in antibiotic activity given that both AGA and AP3 can reduce cytochrome C under suitable conditions? Two explanations, not necessarily exclusive, are apparent. The first has already been discussed; that the D-alanine group of AGA enables this compound to be taken up by a susceptible cell to react at some undefined site, whereas AP3 possesses similar reactivity but cannot access the target. Alternatively, the proposed difference in redox potential between AGA and AP3 may be sufficient to account for the toxic and non-toxic properties of these closely-related phenazine derivatives. For instance, phenazine methosulphate has a standard reduction potential (E'_0) of +0.08 V and is able to accept electrons from cytochrome B ($E'_0 = +0.07$ V) but not from cytochrome C ($E'_0 = +0.25$ V). The ability of AGA to spontaneously oxidize NADPH but not cytochrome C indicates that AGA could accept electrons from one or more of the electron carriers between NADPH and cytochrome C in the electron transport chain, this diversion of electrons would interrupt the respiration chain, and compromise the cells ability to generate energy. In contrast, AP3 appears to be a weaker oxidant than AGA and cannot spontaneously draw electrons from NADPH, which has the lowest E'_0 (-0.032 V) and is therefore the strongest reductant of all the respiratory-chain electron carriers. This finding suggests that AP3 would not interrupt the electron transport chain unless a suitable intermediate such as a redox cycling oxidoreductase was present. It is on this point that the former hypothesis is again invoked. Reductases are present in living organisms, and are even required for the activation of some redox active antibiotics such as metronidazole (Tally et al., 1981). Metronidazole resistance in *Helicobacter pylori* arises from the mutation or reduced expression of two genes encoding nitroreductases (Jeong et al., 2001; Sisson et al., 2000). In an analogous situation oxidoreductases present in a target organism could mediate the antibiotic activity of AGA and AP3 via redox activation as

illustrated in Figure 4.10. Thus, it is likely that within a cell both AGA and AP3 would be able to draw electrons out of the respiration chain, however it is proposed that AP3 cannot enter a cell to take part in such reactions.

Assuming that redox activity is an important aspect of the antibiotic activity of AGA, what types of reactive species are involved in the process? At least two possibilities exist. The first possibility, that AGA can interfere with normal electron transfer processes has been discussed. A second possibility is that AGA could abstract an electron from a substrate such as an oxidoreductase to become reduced; the resulting cation could be resonance stabilized due to the conjugated nature of the phenazine nucleus and subsequently donate electrons to an acceptor such as oxygen to create toxic reactive oxygen species. However, because obligate anaerobes are highly sensitive to AGA it is clear that the generation of reactive oxygen species is not absolutely necessary for antibiotic activity. Additionally, catalase significantly reduced the oxidation of hemoglobin but not the reduction of cytochrome C by AGA, thus it appears that at least two different reactions are possible (Table 4.8). Not only is the oxidation of hemoglobin by AGA slow, but a potent catalase from *S. entomophila* did not provide any detectable degree of resistance against AGA for *S. entomophila* or *E. coli*, suggesting that the antibiotic activity of AGA does not involve H₂O₂. Superoxide did not appear to significantly alter the rate of cytochrome C reduction, and this has been observed elsewhere for phenazine methosulphate (Picker and Fridovich, 1984). In agreement with this, Davis et al (1983) detected superoxide in a system involving NADH reduction of pyocyanin but not phenazine methosulphate. This demonstrates that the generation of reactive oxygen species varies for different phenazines and that superoxide cannot be discounted as a factor in the antibiotic activity of AGA until it has been assessed.

To further assess the influence of reactive oxidative compounds in the antibiotic activity of AGA, the suppression of antibiotic activity by antioxidant compounds could be measured (this could be carried out in *E. coli* strain DE880 which carries a *sulA::lacZ* fusion and responds with great sensitivity to antibiotic activity). For instance, hemoglobin oxidation by acetylphenylhydrazine is almost completely inhibited by the antioxidant ascorbate (Winterbourn, 1985). Alternatively, the generation of oxygen radicals by AGA could be detected and measured using genetic means. In *E. coli*, the induction of specific regulons in response to hydrogen peroxide (*oxyR*, ≥ eight genes) and superoxide (*soxRS*, ≥ 15 genes), can be detected and measured via the expression of *lacZ* gene fusions to promoters from different oxidative-stress induced genes (Gaudu et al., 2000; Kogoma et al., 1988; Michan et al., 1999). Interestingly, the *soxRS* regulon includes *nfsA*, which encodes an oxygen-insensitive

nitroreductase, and *fpr*, which encodes NADPH ferredoxin reductase, both with potential to increase the activity of AGA (Bianchi et al., 1993; Liochev et al., 1994; Paterson et al., 2002). In the presence of oxygen the production of even small amounts of superoxide may lead to the reduction of AGA, which could then react with a member of the electron transport chain.

All of the current physicochemical data on AGA suggests that it has a similar antibiotic mode of action to that proposed for pyocyanine by Baron and Rowe (1982) who hypothesized that the 'compound' diverts electron flow at some specific point in the respiratory chain, bypassing several necessary carriers and starving the cell of energy. Since AGA is only weakly mutagenic and it is active in the absence of oxygen, mutagenesis and the generation of reactive oxygen species probably have only minor roles in the potency of AGA. Thus, direct interaction with the electron transport chain is likely to be a more important factor. The prediction of possible targets from which AGA could divert electrons will require determination of its standard reduction potential.

Chapter 5. SUMMARY AND FUTURE DIRECTIONS

The occurrence of Fire Blight in New Zealand is lower than predicted by established disease forecast systems (Thomson and Hale, 1987). To account for the low incidence of disease it was proposed that the causative agent of Fire Blight, *E. amylovora*, was suppressed in New Zealand orchards due to competition with other organisms. On this basis, the current research program was initiated by Drs Khris Mahanty, Leah Kearns and Chris Hale, and led to the isolation of a potent suppressor of *E. amylovora*, *Erwinia herbicola* strain Eh1087 from an apple orchard in Canterbury, New Zealand (Kearns 1993, Kearns and Hale, 1995). The production of antibiotic by Eh1087 was found to be important for disease suppression in an immature pear fruit assay and led to preliminary investigations into the genetic basis of antibiotic production by Eh1087 and characterization of the antibiotic. These preliminary data provided the basis for the present study, an in-depth investigation into the molecular genetics of antibiotic production by Eh1087.

Research Summary

In this dissertation three key research areas on antibiotic production by Eh1087 have been advanced: i) the significance of antibiotic production in planta has been established, ii) a cluster of genes sufficient for antibiotic production has been substantially characterized, and iii) the chemical structure of the antibiotic has been determined and its mode of action is at least partially understood. Each of these areas has been presented as separate sections of research, however it is worth noting that these research areas were carried out concurrently. This has relevance to the evolution of the present investigation, for instance, results from molecular genetic experiments often provided the rationale and influenced the design of experiments to characterize the antibiotic produced by Eh1087 and vice versa. Thus, there are frequent cross-citations from one chapter to the next. The integration of various scientific disciplines was essential since the results from different research areas are complementary and synergistic. Perhaps most importantly, relationships between antibiotic structure and putative gene functions led to a number of testable predictions – a prerequisite for continued investigation of antibiotic biosynthesis by Eh1087.

The biological significance of antibiotic production

So what were the most significant findings of the present investigation? The present study demonstrates for the first time that the inhibition of *E. amylovora* by Eh1087 holds true in the real world - the stigma of blossoms where *E. amylovora* and antagonists compete for space and nutrients. Because the multiplication of *E. amylovora* on stigma is required for the development of a population sufficient for plant infection, the inhibition of stigma colonization is a potent mechanism for biological control. The significance of antibiotic production in *E. amylovora* suppression in planta and the novel structure of the antibiotic produced by Eh1087 indicates that this bacterium is an excellent biological control candidate that warrants further investigation and development, particularly for use as part of a microbial consortia of biological control strains with different suppression mechanisms.

The AGA gene cluster: current knowledge and future possibilities

A major goal of the current study was to characterize all the genes required for antibiotic synthesis. A 15 kbp section of DNA was shown to be sufficient for AGA production by a heterologous host and became the major focus of the present study. Sixteen genes were characterized from within this region and each was shown to have a role in either antibiotic biosynthesis or resistance. Genes were grouped according to their involvement in the synthesis of a coloured intermediate. These assignments were useful for elucidating the role of each gene in the biosynthesis of antibiotic. Strong evidence suggests that the Group 1 gene products are involved in the synthesis of a phenazine nucleus, similar to the phenazine-1-carboxylic acid (PCA) operon found in some fluorescent *Pseudomonads*. It would be interesting to see whether Eh1087 mutants missing a Group 1 gene can produce AGA when transformed by a plasmid encoding genes for the synthesis of PCA, and vice versa. This is the first time that genes for synthesis of the phenazine nucleus have been described for a non-*Pseudomonad*, and the first time that Groups of genes have been described that are required for modification of the phenazine nucleus. These modifications appear to involve the step-wise conversion of the phenazine nucleus (AP1) to a red intermediate, AP2, which is modified and transported across the inner membrane, where the periplasmic (or extracellular) AP3 is finally converted to AGA by addition of a D-alanyl moiety (Fig 5.1). In *Pseudomonas* spp. the products of different single genes are involved in the modification of PCA to a variety of phenazines. Such genes may provide a useful resource for genetic manipulation of Eh1087 to produce different phenazine antibiotics and enhance the biological control capabilities of this strain, either against *E. amylovora* or against other pests such as fungi that Eh1087 cannot currently suppress. Since

orchard spray regimes often include antifungal sprays, an antibacterial and antifungal strain of Eh1087 could be of great utility to the orchardist (Kearns, 1993).

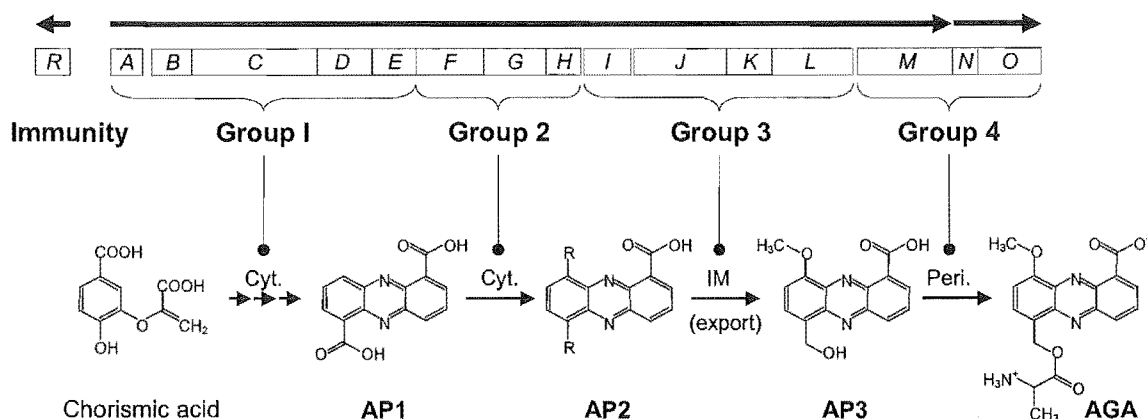


Figure 5.1. Summary model of antibiotic production by Eh1087

Overall model of the data and predictions arising from the present study. *ehp* genes are shown in boxes with heavy black arrows depicting significant transcripts required for antibiotic production. The reactions are given below the groups of genes required for each antibiotic synthesis step. Each modification is predicted to occur in the cytoplasm (Cyt.), across the inner membrane (IM) or in the periplasm / outer membrane (Peri.). The chemical structure of AP1 is unknown but it has similar characteristics as PCA or PDC (shown); the structure of AP2 is unknown; the structure of AP3 is unknown but is likely to be similar to GA (shown) as both can be converted into AGA by the Group 4 gene products; the structure of AGA is known.

As a resource, the collection of *ehp* mutants will be invaluable for further elucidation of the antibiotic biosynthetic pathway. Comparison of the structures of intermediates purified from each mutant would categorically demonstrate the role of each gene product in the synthesis of AGA. Since many proteins with similar sequence to the predicted Ehp proteins are themselves predicted by sequence similarity, these experiments would provide empirical data to strengthen the predictive power of the sequence databases and thereby benefit research projects not directly related to this one.

Information about the regulation of the AGA gene cluster is of great use in the manipulation of Eh1087 to enhance antibiotic production or to increase the range of conditions in which Eh1087 can produce antibiotic. *E. coli* harbouring the AGA gene cluster produce far less antibiotic than

Eh1087. Additionally, expression of the gene cluster during entry to stationary phase decreases in Eh1087 but not *E. coli*. Thus, it is hypothesized that Eh1087 has a positive inducer of antibiotic production during periods of growth that is not present in *E. coli*, and this inducer does not function in stationary phase. Whatever the reason for the expression differences between *E. coli* and Eh1087 the mechanism(s) behind it could provide a good target for genetic manipulation. The manipulation of Eh1087 to produce antibiotic during periods of no or low growth would potentially enhance the ability of Eh1087 to compete against *E. amylovora* that have already colonized a stigma. This is a feature that all current Fire Blight biological control strains lack. In contrast to *E. coli*, the AGA gene cluster conferred on another *E. herbicola* strain the ability to produce nearly as much antibiotic as Eh1087. Thus, the expression of the gene cluster appears to be better in *E. herbicola* than in *E. coli*, perhaps due to general expression differences such as promoter recognition. The possibility that the gene cluster is well expressed in *E. herbicola* and that fact that AGA has different characteristics to antibiotics produced by other *E. herbicola* strains, means that the AGA gene cluster is potentially a good tool for the use in the development of potent new biological control strains. Given that the gene cluster may be horizontally mobile as discussed below it would be best to develop a system to 'immobilize' the AGA gene cluster as far as possible on the chromosome of the new host strain. EhC9-1 is a good candidate for transformation with the AGA gene cluster, as it is currently undergoing licensing for use as a biological control agent.

Origin of the AGA gene cluster

Two interesting questions arise regarding the mobility and origin of the AGA gene cluster. It would be very difficult to design experiments to determine the origin of the AGA gene cluster, although this may become apparent as more phenazine antibiotic gene clusters are discovered. In contrast, it is possible to test the hypothesis that the gene cluster is a horizontally mobile element. Two pieces of evidence suggest that this may be the case; firstly, it is located on a large plasmid, and secondly, it is bordered by putative transposase / integrase genes. The stability of the AGA gene cluster in Eh1087 is an important consideration for the use of this strain as a biological control agent. It is therefore essential that the mobility of the AGA gene cluster between organisms is assessed. If transfer does occur, it would be useful to measure the transfer frequencies in different environments and to investigate the mechanisms involved. Future studies should focus on assessing the possibility of horizontal transfer from Eh1087 to *E. amylovora*, however many other saprophytic bacteria could potentially act as transfer mediators and should also be assessed.

Purification, structure, and properties of the antibiotic produced by Eh1087

Chapter 4 presents data relating to the properties of the antibiotic produced by Eh1087. Initially, media conditions and growth phase were assessed for optimization of antibiotic yield. Subsequently, a three stage antibiotic purification process was developed that enabled the chemical structure of the antibiotic to be determined. The antibiotic produced by Eh1087 was found to be a phenazine compound, D-alanylgriseoluteic acid (AGA). AGA was found to kill a wide range of Gram⁺ and Gram⁻ bacteria, although some species demonstrated inherent resistance or tolerance. The results conclude with preliminary investigations into the mode of action of AGA which revealed that it has bacteriocidal activity, generates an SOS response in susceptible bacteria, is weakly mutagenic *in vivo*, and has redox activity. It was speculated that the redox potential of AGA would enable it to divert electrons from the electron transport chain and inhibit the ability of a cell to generate energy, whilst comparison with AP3 suggested that D-alanine is required for antibiotic uptake by a susceptible cell. Further investigations into the mode of action of AGA should test its ability to interfere with the electron transport chain. Determination of the standard reduction potential of AGA would provide clues as to whether or not this is likely to occur. Additionally, measurement of the change in redox state of the electron transport chain intermediates in the presence of AGA would show which electron carrier(s), if any, can be reduced by AGA and hence the point at which AGA diverts electrons and prevents a cell from generating energy. Similar studies with AP3 would support or refute the hypothesis that the lack of antibiotic activity of this compound is the result of properties other than redox potential, properties that may, for instance, affect uptake by otherwise susceptible cells. The use of defined *E. coli* mutants would be useful to assess the influence of other AGA properties such as mutagenicity, the generation of oxidative species, and even AGA uptake by transport mechanisms. Since AGA is weakly mutagenic and could potentially generate free radicals, the phytotoxicity of Eh1087 in planta should be also assessed. In particular, it is important that blossoms inoculated with Eh1087 are not affected in their ability to produce normal fruit.

The research presented in this dissertation provides fundamental information about the genetic basis for antibiotic production in Eh1087, and in doing this opens up a number of interesting research areas. Notably, the origin of the gene cluster has wide implications for the evolution of organisms in general. In addition, the present study has provided the tools and necessary information for manipulation of the gene cluster to test hypotheses on gene function and regulation, to create new biological control strains, and potentially even new antibiotics.

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APPENDICES

A1. General Materials and Methods

A1.1. Bacteriological methods.

The bacterial strains and plasmids used in this study are described in Table A1. *E. coli* strains were routinely grown at 37 °C, and *E. herbicola* and *E. amylovora* at 30 °C. Bacteria were cultured in Luria-Bertani (LB) medium or 1-A minimal medium with 0.2% glucose as described (Miller, 1972). Antibiotics, 5-bromo-4-chloro-3-indolyl phosphate (XP), and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Xg), were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were used at the following concentrations: ampicillin (Ap), 100 $\mu\text{g.ml}^{-1}$; chloramphenicol (Cm), 30 $\mu\text{g.ml}^{-1}$; gentamicin (Gm), 30 $\mu\text{g.ml}^{-1}$; kanamycin (Km), 50 $\mu\text{g.ml}^{-1}$; rifampicin (Rf), 50 $\mu\text{g.ml}^{-1}$; streptomycin (Sm), 50 $\mu\text{g.ml}^{-1}$; tetracycline (Tc), 15 $\mu\text{g.ml}^{-1}$; XP and Xg, 25 $\mu\text{g.ml}^{-1}$. Enzymes for DNA manipulation were purchased from BRL and used according to the manufacturers instructions. β -galactosidase activity of whole cells was determined as described previously (Miller, 1972). Viable cell counts were carried out by serially diluting bacterial cultures either in microtitre dishes (100 μl per well) or in eppendorf tubes (1 ml per tube).

A1.2. Antibiotic activity assays

Antibiotic activity was routinely assayed on a lawn of *Erwinia amylovora* strain Ea8862 as follows. LB or 1-A minimal medium (supplemented with niacin (Sigma) to 50 $\mu\text{g.ml}^{-1}$) agar plates were overlaid with 2 ml of 0.75 % 1-A or LB agar containing 20 μl of an overnight LB broth culture of Ea8862. Strains to be tested for antibiotic activity were placed directly on the lawn of Ea8862, whereas test compounds were dried onto 6 mm ϕ antibiotic disks (Schleicher & Schuell) which were placed on the lawn. Activity was assessed as zones of Ea8862 inhibition after overnight incubation at 30 °C.

Table A1. Bacterial strains and plasmids

Strain or plasmid	Relevant Genotype or description	Source or ref.
<i>E. coli</i>		
DE880	HfrH $\Delta(lac-argF)U169 relA1 thi-1 cps-3 malF55::Tn5$ $sulA::Mu d(lac Ap) XCam (Mu+) srlC300::Tn10$	Ennis et al. (1989)
DE894	HfrH $\Delta(lac-argF)U169 relA1 thi-1 cps-3 malF55::Tn5$ $sulA::Mu d(lac Ap) XCam (Mu+) \Delta(recA-srlR)301::Tn10$	Ennis et al. (1989)
^b DH5 α	$supE44 \Delta lacU169(\phi 80 lacZ \Delta M15) hsdR17 thi-1 relA1$	Hanahan (1983)
Ea8862	<i>Erwinia amylovora</i> strain isolated from <i>Malus x domestica</i>	ICMP ^a
Eh1087	<i>Erwinia herbicola</i> strain isolated from <i>Malus x domestica</i>	Kearns (1993)
EhehpA-EhehpO	Eh1087 AGA-negative derivatives each with single mini-Tn5lacZ2 insertions in an <i>ehp</i> gene	This study
Eh Δ AGA	Eh1087 derivative in which the AGA-gene cluster has been deleted and replaced with a Ω Sm ^R cassette	This study
HB101	$supE44 hsdS20(r_B-m_B-) recA13 ara14 rpsL20 proA2 lacY1$ $galK2 xyl5 myl1$	Boyer and Roulland (1969)
^b MC4100	$araD139 \Delta(lacIPOZYA-grgF) U169 rpsL thi recA-56 sup^O$	Casadaban (1976)
P678-54	Minicell-producing mutant of <i>E. coli</i> K-12 P678	Adler et al. (1967)
S17-1	$thi pro hsdR- hsdM+ \Delta recA \lambda pir RP4-2 Tp^R::Mu-Km^R::Tn7$	Simon et al. (1983)
WP3101-WP3106	$uvrA155 trpE65 \Delta(lac-pro) malB15 lon-11 sulA1$ F+(<i>lacI- lacZ- proAB+</i>): six <i>lacZ</i> reversion test strains	Ohta et al. (1998)
WP3101P-WP3106P	WP3101-WP3106 containing pKM101 (<i>mucAB</i> , Ap ^R)	Ohta et al. (1999)
Plasmids		
pAGA	pBluescriptKS- containing a 15.5 kbp section of DNA encoding the entire AGA-gene cluster	This study
pBluescriptKS-	M13-, ColE1 <i>ori lacZ</i> α KS-oriented MCS, Ap ^R	Stratagene
pBR322	ColE1 derivative, Ap ^R , Tc ^R	This study
pBB7	<i>Bam</i> HI 7.4 kbp subclone of pLA272 in pBR322, contains <i>ehpR</i> , <i>ehpA-E</i>	This study
pBB7:: <i>ehpR</i>	pBB7 carrying a Tn10-LK insertion in <i>ehpR</i>	This study
pBB7:: <i>ehpA - ehpF</i>	Six derivatives of pBB7 each carrying a mini-Tn5lacZ2 insertion in one of the genes <i>ehpA - ehpF</i>	Kearns and Mahanty (1998)
pBE5a	<i>Eco</i> RI 5 kbp subclone of pLA255 in pBR322, contains <i>ehpG-ehpK</i>	This study

Table A1 continued.

Strain or plasmid	Relevant Genotype or description	Source or ref.
pBE5b	<i>Eco</i> RI 5 kbp subclone of pLA272 in pBR322, contains <i>ehpM-ehpO</i>	This study
pBSEC1	pBR322 with a 13 kbp DNA fragment from <i>S. entomophila</i> BC4B encoding a catalase gene	Giddens (1995)
pBSEC1-1	pSEC1 containing a miniTn10 insertion within a catalase gene	Giddens (1995)
pEhpR	pBluescriptKS- containing a 1.8 kbp DNA fragment encoding <i>ehpR</i>	This study
pHRP315	IncQ plasmid containing an Ω Sm ^R cassette	Parales and Harwood (1993)
pJQ200mp18	Gm ^R , MCS, contains <i>sacB</i> (sucrose sensitivity)	Quandt and Hynes (1993)
pLAAAGA	Sections of DNA from upstream and downstream of the AGA gene cluster cloned either side of a Ω Sm ^R cassette	This study
pLAFR3	RP4 (IncP-1) ori, λ cos, pUC9 MCS and <i>lacZ</i> α^+ Tc ^R	Staskawicz, et al. (1987)
pLA272	AGA ⁺ , AGA ^R cosmid from Eh1087 genomic library	Kearns (1993)
pLA272:: <i>ehpA-ehpO</i>	Derivatives of pLA272 each carrying a mini-Tn5 <i>lacZ2</i> insertion (O1 or O2) in one of the genes <i>ehpA-ehpO</i>	This study
pLA <i>recA</i>	A cosmid containing the Eh1087 <i>recA</i> gene	This study
pLA <i>recA</i> :: Ω Cm ^R	As for pLA <i>recA</i> , however the <i>recA</i> gene is interrupted by a Ω Cm ^R cassette	This study
pKS <i>recA</i>	A 1.8 kbp <i>Pst</i> I fragment of DNA containing the Eh1087 <i>recA</i> gene cloned in pBluescriptKS	This study
pKS <i>recA</i> :: Ω Cm ^R	As for pKS <i>recA</i> , however the <i>recA</i> gene is interrupted by a Ω Cm ^R cassette	This study
pUT::mini-Tn5 <i>lacZ2</i>	mobilizable suicide delivery plasmid containing <i>tnp</i> for transposition	de Lorenzo et al. (1990)
pUT::mini-Tn5 <i>phoA</i>	As for pUT::mini-Tn5 <i>lacZ2</i> ; for transposition with mini-Tn5 <i>phoA</i>	de Lorenzo et al. (1990)

^aICMP; International Collection of Micro-organisms from Plants, Landcare Research / Manaaki Whenua New Zealand Ltd., Auckland, New Zealand. ^b*recA*⁺ derivatives of these strains were created by P1 transduction of *recA* from *E. coli* W3110.

A1.3. Manipulation of DNA

A1.3.1. Total DNA Preparation

Genomic DNA was prepared by the method of (Pitcher et al., 1989). Cells were collected in an eppendorf tube by centrifugation from one ml of a bacterial culture grown for 18 h. The cells were resuspended in 100 μ l TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8), lysed with 0.5 ml GES reagent (5 M guanidium thiocyanate (Sigma), 100 mM EDTA, and 0.5 % v/v sarkosyl), and vortexed until fully mixed. The cell lysate was cooled on ice and the denatured proteins precipitated by adding 0.25 ml 7.5 M ammonium acetate and placing the tube on ice for 10 min. 0.5 ml of a mixture of chloroform and isoamyl alcohol (24:1) was added, mixed thoroughly by vortexing, centrifuged (10000 g, 10 min.), and the aqueous phase was transferred to a new eppendorf tube. DNA was precipitated by the addition of 0.54 volumes of cold isopropanol, and collected by centrifugation (10000 g, 1 min.). DNA was rinsed three to five times with 70 % ethanol, dried, and dissolved in 50 μ l TE buffer.

A1.3.2. Plasmid DNA Preparation

Generally, plasmid DNA was isolated from bacteria using the alkaline lysis procedure (derived from Birnboim and Doly, 1979; Sambrook *et al.*, 1989). Cells cultured for 18 h were resuspended in an isotonic buffer (1 % glucose; 25 mM Tris-HCl; 10 mM EDTA, pH 8.0). The buffer was adjusted to 0.67 % SDS and high pH (0.67 M NaOH) to lyse the cells and denature proteins, before the lysate was neutralized with 1 M sodium acetate (pH 4.8) to precipitate the denatured components, which could then be sedimented by centrifugation (10000 g, 10 min.) and discarded. Plasmid DNA was precipitated from the lysate by the addition of two volumes of ethanol, and collected by centrifugation (10000 g, 5 min.). Plasmid DNA was rinsed twice with 70 % ethanol, and purified using one or more of the following steps if required. High Mw RNA was precipitated from the plasmid DNA solution after adjusting the solution to 2.5 M LiCl, and removed by centrifugation. The remaining low Mw RNA was digested with RNaseA (100 ng.ml⁻¹) at 37 °C for 15 - 30 min., and removed along with other proteins still present in the plasmid DNA solution by phenol – chloroform extraction. Plasmid DNA was collected from the aqueous phase after adjusting it to 0.25 M potassium acetate, adding two volumes of ethanol, and centrifuging the plasmid precipitate. Plasmid DNA was washed twice in 70 % ethanol, dried, redissolved in an appropriate volume of TE or dH₂O and stored at -20 °C.

A1.3.3. Restriction enzyme digestion of DNA

DNA molecules were digested with restriction endonucleases (and *Bal31* exonuclease) purchased from Gibco BRL according to the manufacturers recommendations. When more than one endonuclease was required, the reactions were either carried out in the most suitable buffer or the DNA was digested with one endonuclease, precipitated, digested with the next endonuclease, and so on. To partially digest DNA, restriction endonuclease were suitably diluted, and the reactions were incubated for 15 min. at the appropriate temperature and stopped by cooling on wet ice. Digested DNA was purified by phenol – chloroform extraction before further manipulation.

A1.3.4. Separation of DNA fragments by agarose gel electrophoresis

Digested DNA fragments were separated by electrophoresis through a gel composed of agarose dissolved to an appropriate concentration in 1 x TAE buffer or in 0.5 x TBE for FIGE electrophoresis using a FIGE Mapper™ (Bio-Rad). Separated DNA fragments were stained with ethidium bromide and visualized on a Sigma T2210 UV transilluminator (302 nm) or with an Ultralum KS3000 gel Visualization-Documentation and Analysis system.

A1.3.5. Elution of DNA from an agarose gel

DNA fragments were eluted from agarose gels using a Prep-A-Gene® DNA purification kit (Biorad) according to the manufacturers instructions.

A1.3.6. Dephosphorylation of DNA to prevent self-ligation

Linearized plasmid DNA molecules (usually vector) were treated with Calf Intestinal Phosphatase (CIP, (Boehringer Mannheim)) to remove terminal 5' phosphate groups and prevent self ligation. Generally 1 µg of linearized plasmid DNA was treated with 1 unit of CIP at 37 °C for 30 min., before being purified by phenol-chloroform extraction.

A1.3.7. Ligation of DNA fragments

Linearized DNA molecules were ligated using T4 DNA ligase (Gibco BRL) in a ratio of ca. 1-5 µg DNA to 1 unit ligase at room temperature for 2-16 hr.

A1.4. Transformation of bacteria with plasmid DNA**A1.4.1. Calcium chloride method.**

E. coli strains were grown in LB at 37 °C with vigorous shaking to an O.D₆₀₀ of 0.4, and harvested by gentle centrifugation (5 min., 4000 rpm, 4 °C). The cells were rinsed once with 10 mM NaCl, and twice with 100 mM CaCl₂. Cells resuspended in CaCl₂ were stored on ice

for 20 min. before plasmid DNA was introduced and the cells stored for a further 30 minutes on ice. Cells were then exposed to 42 °C for 60 - 90 s, placed on ice for 60 s, before ten volumes of LB was added and the cells elaborated for 60 min.

A1.4.2. Electrotransformation.

Generally *E. coli* strains were grown in LB with vigorous agitation to an OD₆₀₀ of 0.3 to 0.5, and *E. herbicola* or *E. amylovora* strains were grown overnight. Cultures were chilled on ice for 2-10 min., before cells were rinsed three times in cold sterile dH₂O by the process of centrifugation (5 min., 4500 rpm, 4 °C), supernatant removal, and resuspension in dH₂O. Cells concentrated ca. 100 times were divided into aliquots of 40 µl for immediate use, or were finally resuspended in 10 % filter-sterilized glycerol before storage at -80 °C. Electroporation was carried out using a Gene Pulser™ (Bio-Rad) set with capacitance at 25 µF, pulse-controlled resistance at 200 Ω, and voltage at 1.8 kV for 0.1 cm gap cuvettes (Dower et al., 1988; Zabarovsky and Winberg, 1990).

A1.5. DNA hybridization

A1.5.1. Colony transfer

Single colonies of an Eh1087 genomic library constructed in a previous study (Kearns, 1993) were transferred onto 2 % LB agar plates supplemented with tetracycline (15 µg.ml⁻¹) in grid formation using a numbered template, and overlaid with circular Hybond N+ membrane filters. After incubation at 37 °C overnight the filters were removed and the plates were stored at 4 °C until needed. Cells attached to the membranes were lysed by placing the membranes colony side up on Whatmann 3MM filter paper soaked with 10 % SDS for 3 min. To fix DNA to the membranes they were rinsed with freshly prepared denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 5 min. The filters were finally rinsed twice with fresh neutralising solution (0.5 M Tris pH 8.0, 1.5 M NaCl), placed on a paper towel and air dried.

A1.5.2. Southern transfer

DNA was transferred from an agarose gel to a Hybond N+ membrane using a Pharmacia LKB VacuGene XL vacuum blotting system as described in the supplied instruction manual. The DNA was partially depurinated by covering the gel with a layer of 0.25 M HCl under a vacuum pressure of 50 mbar for 10 min. The HCl was removed by aspiration, the gel covered with 0.4 M NaOH, and the DNA was transferred at a vacuum of 50 mbar for 1 hr. After this period the membrane was rinsed in 2 x SSC and air dried on tissue paper, and the gel was restained in ethidium bromide to determine the efficiency of transfer. Membranes were either

probed immediately or wrapped in Glad Wrap™ and stored at room temperature until required.

A1.5.3. Hybridization detection using ECL™ (Amersham)

Fragments of DNA to be used as probes were labeled with the Amersham Enhanced Chemiluminescence (ECL) direct nucleic acid labeling and detection system according to the manufacturers instructions. Hybridization was carried out using a rotary mini-hybridization oven (Hybaid) in which the membrane bound DNA was exposed to 20 ml of ECL™ gold pre-hybridization buffer (0.5 M NaCl, 5 % w/v blocking reagent) containing labeled probe at 10 ng.µl⁻¹. Following hybridization, unbound probe was removed by rinsing the membrane at 55 °C for 20 min. with 50 - 100 ml of a solution containing 0.4 % SDS and 0.1xSSC, followed by two rinses with 100 – 200 ml 2xSSC for five min. at room temperature. To detect hybridized probe DNA, the rinsed membranes were exposed to equal volumes of detection reagents 1 and 2 for 1 min., wrapped in Glad Wrap™, and exposed to Amersham Hyperfilm-MP in an autoradiography cassette for 30 sec. to 16 hr depending on the strength of the signal. The films were then immersed in AGFA G-150 developer for 5 min., washed in running water, and immersed in AGFA G-334 fixer for a further 5 min. The autoradiographs were finally rinsed in water and air dried.

A1.6. Protein Electrophoresis

Protein preparation and polyacrylamide gel electrophoresis (PAGE) was carried out essentially as described by Hames and Rickwood (1990). Denaturing polyacrylamide gels (0.75 mm thickness) for SDS-PAGE, and molecular weight standards (Biorad) were prepared according to the manufacturers instructions. Gradient gels were prepared using a gradient former (BRL). SDS-PAGE was carried out using a Biorad protean II gel apparatus, a Biorad Buffer recirculation Pump, and a Biorad PowerPac 1000. Protein samples for separation were resuspended in an equal volume of 2 x treatment buffer (0.125 M Tris-HCL (pH 6.8), 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, and 0.25 % w/v bromophenol blue), boiled for 3 min., loaded onto a polyacrylamide gel and separated by electrophoresis with a constant current set at 13 mA per 0.75 mm gel until samples reached the boundary between the stacking gel and the resolving gel, and then at 18 mA per 0.75 mm gel until the run had completed. Gels were stained with Coomassie G-250 blue for 90 min., destained in 50 % methanol : 10 % acetic acid for 1 hour, and then destained overnight in 5 % methanol : 7 % acetic acid : 3 % glycerol. Destained gels were either photographed, or, for radiolabelled proteins the gels were exposed to 500 ml Amplify Fluorographic Reagent (Amersham) for 30

min., before being rinsed in dH₂O, placed on a sheet of Whatmann no. 1 chromatography paper and dried under vacuum at 65 °C using a Model 443 slab drier (Biorad). Dried gels were exposed to Amersham Hyperfilm-MP in an autoradiography cassette at -80 °C for 1-7 days depending on the strength of the signal.

A1.7. Sequence Data

A1.7.1. DNA Sequencing

DNA sequence determination and analysis. DNA sequence was determined by the Sanger method using chain terminating dideoxynucleoside triphosphate; infrared dye (IRD40) labeled sequencing reactions were resolved on a Licor 4000LD IR2 automated sequencer, and Rhodamine-dye terminator labeled sequencing reactions were resolved on an ABI 377XL automated DNA sequencer. Primer sites throughout the regions to be sequenced were provided either by transposon insertions, by subcloning of the regions to pBluescript KS- (Stratagene), or by designing specific primers to known DNA sequence. For the *ehp* gene cluster, the DNA sequence of both strands was compiled manually, and both strands were aligned for authentication.

A1.7.2. DNA sequence analysis

Gene predictions were created using the Heuristic GeneMark.hmm gene prediction program (15 June 2001, <http://dixie.biology.gatech.edu/GeneMark/heuristic.cgi>) (Lukashin and Borodovsky, 1998). DNA sequence was compared with the GenBank DNA sequence database using the BLASTN and BLASTX programs (<http://www.ncbi.nlm.nih.gov/blast>) (Altschul et al., 1997). The predicted gene products of the AGA gene cluster were analyzed for conserved domains using PROSITE (25 July 2001, <http://www.expasy.ch/tools/>) (Hofmann et al., 1999) and Reverse Position Specific (RPS) BLAST version 2.2.1 (28 August 2001, <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Altschul et al., 1997), for signal sequences and their cleavage sites using the SignalP V2.0 program (28 August 2001, <http://www.cbs.dtu.dk/services/SignalP/>) (Nielsen et al., 1997), and for membrane regions using the Dense Alignment Surface (DAS) method (25 July 2001, <http://www.sbc.su.se/~miklos/DAS/>) (Cserzo et al., 1997), and the transmembrane hidden Markov model (TMHMM) version 2.0 program (28 August 2001, <http://www.cbs.dtu.dk/services/TMHMM/>) (Sonnhammer et al., 1998). Promoter predictions were carried out using the Neural Network Promoter Prediction for prokaryotes (23 October 2001, http://www.fruitfly.org/seq_tools/promoter.html)

A2. DNA Sequence Data

A2.1. DNA sequence and predicted translation products of the AGA gene cluster

DEFINITION: D-alanylgriseoluteic acid synthesis gene cluster, complete cds.
 ACCESSION: AF451953 (bankit434340)
 ORGANISM: *Pantoea agglomerans* (Erwinia herbicola) strain Eh1087
 SOURCE: Bacteria; Proteobacteria; gamma subdivision; Enterobacteriaceae;
 Db-xref: taxon:549
 REFERENCE: 1 (bases 1 to 14924)
 AUTHORS: Giddens, S. R.
 TITLE: Characterization of a novel phenazine antibiotic gene cluster in
Erwinia herbicola Eh1087
 JOURNAL: Submitted (27-NOV-2001) Plant and Microbial Sciences, University
 of Canterbury, Ilam Road, Christchurch, Canterbury 8004, New
 Zealand

Translated sequence data:

For each gene the start codon = 1, and the predicted product has the same name as the gene.

Gene: **ehpR**

CDS: reverse complement [390 - 1]

translation:

MTDLAGPTITPNLQLVYVSNVERSTDFYRFIFKKEPVFVTPRYVAFPSGDFALFAIWSGGEEPVAEIPRFSEIGIMLP
 TGEDVDKLFNEWTQKSHQIIIVIKEPYTDVFGRTFLISDPDGHIIIRVCPLD

Gene: **ehpA**

CDS: complement [809 - 1285]

translation:

MYLTDEDAIRIREINRQVVSQYLSSTRGIARLKRHELFAEDGEGGLWTTETGEPPIIRGIENLEKHAKWSLECFPDWE
 WYNIKIFTTDNPDHVVVECDGRGLIRFPGYPESYYENHFHISFELRNLIVRNREFMNPVTQLKCLGIDVPRIKREGI
 PS

Gene: **ehpB**

CDS: complement [1419 - 2048]

translation:

MYRTLKSESYSLSNIIIPDEQPLAWNIEPQRSALLVHDMQRYFVDALPCEPVDTVIHSISLILKWARINEIPVFYSAQ
 PGGMTPEQRGLLNDLWGPGRMTTEDEKIIITELTPSPGDTVLTWKWRYSAFYRSPLAEMLKNDQRSLIITGIYASVGI
 SATAIDAFTQDIKPFVTADGIADFSLAGHQQAINYLADNCAKIISTDGVVNNV

Gene: **ehpC**

CDS: complement [2041 - 3945]

translation:

MSDSLTHILSQPHKPYALLYRPAVSMDSVEILHLTCGKTHSLDKALSADSHHGKLLVVPFCQVAKRGYHAINDETPIL
 TMPITARQSLPLSEVISRLPDLPISVTNTNFNLDDIQFGQRISQLIEHEIGQGAGSNFVLHRKLRTSLVDYHPEQLLS
 LFRLLQTESSAYWCFLINTGDEAFIGVSPPELHASLDNGEMCMNPISGTLRYPEQGETIEALLEFLTNQKETNELYMV
 VDEELKVM SRLCERGAQVSDLRLKQLSQVIHTEYVLKGKTKASISDILTETLPAPTIIGSPVQNACQVIARYEPEGRR
 YYSGVVALVDGTHDNPRLDSAICIRTAETADGKVEIGVGATIVRDSVPLDEAEETRSKAQSLYAALTQDSLPAKKKV
 TPASLKKLKGDNPKVRPLLNERNNRISKFWLSDPEKRNRIYSFADKKILILDGNDAMFTLTFSSLGALAHVEK
 VCSGIDLQGWDLVVAGPGPNPLDVNDFRVNAMREAIKMRATGQPPFAVCLSHQLLCLQLGLPVARLSPPNQGVQKE
 ILLDRNLETAGFYNTFCASINAVTHSQMRQRGIEVYSDPDNGNVHALRAHSFSSVQFHLESVLTNLNGQALLERFVAPL
 FQKNYSGMAS

Gene: **ehpD**

CDS: complement [3945 - 4808]

translation:

MEGYQVDYTVLNAFTHSPLGNPVAVFHDTEKLSEAVMQAIARQLQLSESTFISEKTVDGQARVRIFTPVNELPFAGH
PLMGTAAYSRRHGLDQFVFHTALGAVSISVEHHTETLSTVKIEVPLARTVPFKEEKRLKALGLEQSVLPVEMYDAG
ARHVLVSVDSIETLRELRPDQVLAEEFNLAVNCFAWCGFQAENRMFSPAYGVKEDAGTGSVVGPIALHLMKHGQLKA
NEKIMIEQGILLNRRVCVMYGEIKQENEIASHIELSGQVTLFSDCRALLSNSGK

Gene: **ehpE**

CDS: complement [4846 - 5472]

translation:

VKRLESLTGTTDLFPQYDAPPVVPYEILHNWMLAHHENVREPDATLSTRSDAGMISMRTIFPVRMNGEDLWCATH
LGSRLSLDMCANKAVSCHIIYWRELGRQVSLTGNALLSDDIADQIWHSRASAYDPVSVASHQSEPLNDLTTLQAKIAG
IGCGKLERPERFVVGWGLAFDTYEFWSASSSRIHKRLLYTRNELGWKHERLQP

Gene: **ehpF**

CDS: complement [5453 - 6553]

translation:

MKDYSLEIDAVMKAQINDTNNFVQALMRWHFSKETGSPFWLGMREQNFDPIKDVKTINDLRQFSDISHCLRQEPVA
NLVPQGLPADSHPOVYESGGTTGAPKYVVAYDAWIEALISWRMSGYQHRPGRPSGNTLAAIPTGPHIVGAINKERALR
LGGMFFSIDIDPRWVKRSLSEGDTATVRKYTHHLVDQVQNTLMNQDIRFLVTTPVLRRELLKRPEVVLQMKQSLAQIT
LGGTELNLDEIKFIASEILPDCEFSASYGSTSALGVSRSLITSESQQVIYDSFSPFITDYDVVDSITAQTVEYGERGN
VIVTHLSPWAFYPRVAERDTAIRLPGVSGFAGDRLADIEPLKISEGRKVIIEGVY

Gene: **ehpG**

CDS: complement [6553 - 7488]

translation:

MPNIAQSCPHVNANELPVISAITPLCSPQFLRQREVNIYEKPIALLQQSPGVFADYVIAHLRKSPLVDRASRQKALQ
QAGELFAGSVLCGMNLTEYNTKVSSLTGLPRTVVENASDKIAQSIHALTFALKGIPLQENQATDRYFCAQSVRRGDV
LTVIAPNGTGVHALWPQAIALGYRVVIRPSEREPTAQRVVPAMVAAGLQDYVTLPCNYDVVETLIDAADLSLIYG
ADEITARYADRRNILVQGPGRSKVVGADYPLEKALELIFTSVVGLGGAACVCASSVLVEGDAAELVGNALFCPIK

Gene: **ehpH**

CDS: complement [7485 - 7961]

translation:

MKDPKQREEWLTHFSPQRYSWWCGQVDNYASALVETPEVFKSTQNTYQVMPLVMQASAINDPVQLELPVAAVTFLSF
KEGDNLDLAPALVVTVASGNKNLINAIAINVPGVKNFYIGQVPTVWMHPDVPHDGYLAEFMLQTRGYCVNSSEDEHSK
VT

Gene: **ehpI**

CDS: complement [8006 - 8689]

translation:

MKVVIIANMIKLISQGVYQGHLSVLEISVVPWDIGQAQPAVCDIFDKENAGRLLDVGCGLGLNAHAAAKRGFQVTA
LDSSSAAIQKCKNEGMSDVRFLIASASNTGLDETFDIIILDSALYHALPYDERLTYLKEMRRLAHENTRMHIITFLPAK
NGMPVPLAIHLSEICSAENAGWDVISVDRVEYKNAQAIADFCKKKNLTLTDEVGFTRLPCWHVVFNIR

Gene: **ehpJ**

CDS: complement [8713 - 10149]

translation:

MLRNKMAFFGLCLGFFIIMDDTTTVP LIYTSIINEYQVTPAMAAVWNSYLITYAGFLLFGGRLGDAINRKTVLYYAL
IILAIGALLSGSGQTLIQVILGRALMGVGAGLLTPQSMAFISIIFTQGGRTALGIWGA VAGIATATGPVVTQIFLQT
IGWRWIFIYIPVALIALIFAVICLPGTPGNGAKSKELLSNAIAGIALAAIILGLELLNGMQGMTTGLLLLLSGILL
FAFMLHGELKNHKQYLLPSSLWKDRAFLRTCLISGLLGFLTA FYLPLAYLIELRMGFGPVAISVIMVTIALANALVG
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GRAAAFFNVVRQMMSALGSVAIAIFFDYLLHNSEKRSFTNENSLFSSGSPSVAFASFGCFLIITGLLGLGAWLSRTYP
VVQSVIKETK

Gene: **ehpK**

CDS: complement [10149 - 10937]

translation:

MNTWLISGCSNGLGRCWTESVIHEYGDRVIGITRSVEAAQEMTALYPEHFIPCIADVRDEEYLARILPQLAKDFGIPS
RVVTSAGYAFGTLEDLSTEQLRQQFATNVEGTLMYKPELPLMRQLDEARILLVSSMSGVACWPLLGAYQMSKHAEIA
MGQTLRHELEGTSIQVGVIQPGPYRTGWATTSAQRQPIGSEYDGEALKKRATCGFNIEDPKASLPYFWRMFNTPEMPE
MLATSAKFVDLICAQAEKVKKWRASLA

Gene: **ehpL**

CDS: complement [10948 - 12084]

translation:

LRRWQKAVDILKSSWSEKEQQDSLKQGAQEVLCGSPLLRILDESPLYETGTLREVAVCRELGKTDASLGWLGVVANS
AWSMRANFSLPEGVSEDMRENAMLSMVLGRPGKLNAAADGGWYLNKSWKYASGYPCSSHFFCLAATPSGEVRVVIVP
SNQLIAVADWQSTGLRGTSVTVVEARDINIPKAFTVAYSPILSGNALSYSGKGSYARYFTGVLMNCLPATLLGATESG
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GCVDIVQALLWLRGSSALEKNSPLEKIWRDENVGARHGGFAKLVPPELEGISALKGELSSLTQMY

Gene: **ehpM**

CDS: complement [12155 - 13636]

translation:

MMQKLTPRQVRTKLKTINDSINHMLRDNSEAMAVNDGETSLTWKQMYERAVEIIRYFDKAGMLPGQRLALDAPRSIEL
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GYYKINGFRVDPLEIEEFLQSI PGVFEAVVWMEESSTAPALLKACVNVSRGNLSTRDLRRACMTMSPWLRPAQYLII
SQNEWPIINSRGKSDRAEIKRKFYGV

Gene: **ehpN**

CDS: complement [13626 - 13853]

translation:

MECKQKGHVIVEEIKYMLKEMDVRMDNFTDLGGNSIMAMII TDNLQKKYSINIELAQLLGSKIGEIELKPLGK

Gene: **ehpO**

CDS: complement [13995 - 14924]

translation:

MAASAARNALQFHLSSVDVGFIVAGFSGVPDFIGIDLACQVGAE LNQNQIRTLNLVEGCATAVSVWKHASSLCTEMP
EGKFGLVFLAQRMSDTHQDRFGLMNAALSDGAVACVVGKAITYADQPGLRYISTEDISDCRYVDMMRIEYGGGHLPL
PEGRDSKKDKLGRERIMDNYCFSSQDLNLFILRENNSINVIRRVLEKSVSLDTPPFLHLETKQSIENLCNRIGIP
IERSNISLLSELGHVGCADPLLSFRLMMQRGII SPGSEIVMSTISTGMKWGASLFRYETAISKDNVSYKPERSNN

Figure A1. The complete DNA sequence of the AGA gene cluster

BASE COUNT 4076 a 2982 c 3543 g 4323 t

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 8881 tcttctgtgt ttggcggctg acttgggtgat gcaataaatg gtaaacgggt gctttactat
 8941 gctctcatta ttcttgctat tgggtgcatta ctocagtgtt ctggtcaaac cctgatcacg
 9001 gtcattcttg ggcgtgcgtt tatgggggta ggggctggac ttttgacgcc gcaaagcatg
 9061 gcctttatth ccatcattht tacacaagga ggacgcggta ctgctctggg tatctggggg
 9121 gccgttgctg gcatagcaac ggcaaccggc cccgttggtt cccagatatt tctgcaaaaca
 9181 atcgggtggc gatggattat ttttatttac ataccctgg cgctgatcgc actaatattt
 9241 gctgttattt gtcttcccgg cacaccgggt aacgggtgta aaagcaaaga attgctaagt
 9301 aatgcataag cgggcatttg gctcgtgcga ataattctgg gattagagct tcttaacggg
 9361 ggcatgcagg gaatgaccac agggctgctt ttattattat ctggattttt gctcttcgct
 9421 ttcatgctgc acggtgaatt gaaaaatcat aagcagatc ttttaccatc ttcgctctgg
 9481 aaggatcgag ctttcttgcg tacatgtttg atttccggtt tactcggctt tggcctgaca
 9541 gcgttctacc tgccgctagc ttatttgatt gagctgcgta tgggtttttg accagtagcg
 9601 attagtgtca tcatggtgac aatcgtctct gcaaacgcac tgggtgggtc atttgccgga
 9661 cacttctcgg ataaggcggc tccagaaaca ctgattcgtt gggggatgtg ctgcttcgca
 9721 gctgccacca tgattattgg tattaccgga ttgatgactt atgggtggatc tgtagctttt
 9781 attattatta ttaccgcaat ggctcttgca ggtatcggga cgggcctggc atttgcccc
 9841 ttagctaata aggtcttcag ccgtgcaacg tttaacaatt ttggccgtgc agcgcctttt
 9901 tttaacgtgg tgccgtcagat gatgagcgca ttgggttagt tagcaatagc catatttttc
 9961 gattatctgc ttcataactc tgaaaaaaga agtttcacga atgaaaattc cttattttca
 10021 tctggctctc catctgttgc ctttgcctta ttcggctgct tcctgattat taccggatgc
 10081 ctgggtttag gtcctgggtt atcacggact tatcctgtcg ttcaatcagt tattaaggaa
 10141 accaaataat gaatacctgg cttatcagtg gatgttcaaa cggactaggt cgatgctgga

10201	cggagtctgt	tattcatgag	tatgggtgac	gtgtgattgg	aattaccogt	tcagtagagg
10261	ctgcacaaga	gatgacagct	ctttatccag	aacattttat	tccttgtatc	gctgatgtac
10321	gagatgaaga	gtatctggcc	cgtatactac	ctcaactggc	taaggatttc	ggatataccga
10381	gtcgggtagt	aacgtccgcc	ggctatgctc	agtttggcac	acttgaggac	ctttctacag
10441	aacagttgog	acagcagttc	gctaccaatg	tagaggggac	tctaattgtat	aaaccagagc
10501	taccgctgat	gcgtcagctg	gatgaagctc	gcattctact	ggtgtcaagt	atgagtggcg
10561	tggcgtgctg	gcctcttctg	ggagcttata	agatgagcaa	gcattgcgac	gaagcgatgg
10621	gacaaaccct	ccgtcatgaa	cttgaaggaa	cgtctataca	ggttggcgta	attcagcctg
10681	gaccttatcg	tactggctgg	gcaacgactt	ctgocgcaacg	ccagcctatt	ggttctgaat
10741	atgatggtga	ggcattaaaa	aaacgagcca	cttgtggttt	taacatcgaa	gatacctaaag
10801	ctagcttgcc	ttactttctg	aggatgttta	acacacctga	gatgcctgaa	atgcttgcaa
10861	cgtcagcaaa	gtttgttgac	ctgatttgcg	cgcaggcaga	agagaaagtt	aaaaaatgga
10921	gagcttctct	tgtctaagaa	taatgatgtg	ctcagacgct	ggcaaaaagc	tgtcgatatt
10981	ttgaagtcgt	cttggtcaga	aaaagaacag	caggattctt	taaaagcaag	tgtcaggaa
11041	gtattaaagg	gttcaccact	gttgcgcatc	ctggacgaaa	gcgatctgta	tgagactggg
11101	acgctacgtg	aggtggctcg	tgtatgtcgt	gagctcggaa	aaacagatgc	ttctcttggc
11161	tggcttgtag	gtgttgcgaa	ttccgcttgg	tcgatgcgog	ccaatttttc	tcttcttgag
11221	ggggtgagtg	aagacatgog	tgaaaacgoc	atgctatcaa	tggatttagg	ccgtcccggg
11281	aagcttaacg	ctgcagccga	tgggtcaagg	tggatcttga	acggtagctg	gaaatatgca
11341	tcaggctacc	cctgtttctt	acattttctt	tgccctcgct	caactccatc	tggagaagtg
11401	cgtgttgtea	ttgttcogtc	aaatcaattg	attgctgtgg	ctgattggca	atcaaccggc
11461	ctgagaggaa	cacaaagcgt	gaccgttgag	gcaagggaca	tcaacattcc	taaaagcctt
11521	actgttgcat	attcaccaat	tctttcagga	aatgcgcttt	cgtatagcgg	aaagggctct
11581	tacgcacgct	actttaccgg	cgtattgatg	aactgtcttc	ccgccacact	cttgggagca
11641	acagaaaagc	gcacacagatt	agtacttaat	gaatttaacg	ctcccatatg	tggcagttct
11701	tataacgcta	tgagtgatcc	tgggtgctga	cgttatgaga	tcggacggct	ctgttcaacc
11761	ttcgatttgc	ttgtgcgggc	agccgaatac	aacgcctcaa	ttattgatag	cgcagcgcaa
11821	tgaggcagga	tgtctgaaag	tgaagaacga	gtagcgattc	gcggccgggc	tactcagata
11881	atgcgaggtt	gtgttgatat	tgttcaggcc	ttgttgtggc	tgcgagggtc	atctgcctta
11941	gaaaaaaata	gccctctgga	aaaaatctgg	cgtgacgtaa	atgtcggggc	acgacacggt
12001	ggcttcgcca	aacttgtgoc	agaggagcta	gaagggataa	gtgcattgaa	gggagagctt
12061	tcttctactga	cacaaatgta	ttgacatatt	ggctctgtag	ggcgttggtta	ctgcgacagc
12121	gccactacac	aaacaattat	aggcgagcag	gaatatgatg	cagaaattaa	ccccaggca
12181	agtcgacaca	aagttaaaga	ctataaatga	ttcaataaat	catatgctga	gagataattc
12241	ggaagctatg	gcggtaaatg	atggtgaaac	tagcttaacg	tggaaagcaa	tgtatgagcg
12301	tgcagtagaa	atcataagat	attttgataa	agcgggcatg	ttgcctggcc	agagacttgc
12361	tcttgatgog	cctaggtcta	tagaattata	tctgatgggt	ttagcatgcc	tgtatccgg
12421	gattttcattt	atttctgtac	caagaaatat	gaataagcag	caaaaatacc	agtttgctat
12481	aagcataggt	tgtcagggaa	tctgctcgtc	attatgcaaa	tttgaggaat	tacaaagcac
12541	ttacattggc	gagtgggtgc	ttgggtgtaac	aacccgagaa	tgtagagcct	catatagcaa
12601	tgaggtgtat	tgtgtaagga	catctggtac	cactggagaa	cctaaacttg	taccaattca
12661	catcgctcag	gttaatgcat	ttttgcagaa	tacctatagc	gaaatcccg	taaagaaaaa
12721	aattaactgg	tcgtggatcc	acgatctaac	ttttgacttt	tctatctggg	aattgtttgg
12781	agcactgagt	tatggtggat	gcctagtagt	tatttagtgaa	cagataaaac	ttgatccgac
12841	caaaactcgt	gagatattgg	agaaatctaa	tgttcacttg	ctatctgtca	cgccctcaga
12901	atttcgctac	atatttggtg	gccagttctc	ggcgattttt	aaaaaattat	gtcttaagca
12961	gatgggtgtt	tgccggcgaga	aactgacagc	tgaaactctt	cgtgtatttt	ttcccatctt
13021	taatgagctt	aaggttcaat	tgtggaatac	ttacggccct	tcagaagcaa	ctgttttttg
13081	ttctgcctgg	aaggtagctc	aggacgactt	aacctacgat	attataccaa	ttggcaagcc
13141	atttccgggt	atggtatttt	cttttagagga	atgcaggaaa	gaagggagtg	ggaatctagt
13201	attacaaggg	gggcagggtt	tttccgggtta	tgaaggccgc	gacctatag	ttgctgggtta
13261	catgacggga	gataatttgc	gcagcgataa	cgatggtgta	tggcactata	taggtcgcaa
13321	tgaaggctat	tataaaataa	atggcttccg	agttgatcct	ctggagatag	aggagtttct
13381	tcaactctata	ccgggtgtat	ttgaggctgt	agtatggatg	gaggaaatct	ccacagcgcc
13441	ggcactatta	aaagcttgtg	ttaacgtttc	gagaggaaat	aatctaagta	ctcgtgatct

13501 tagacgcgct tgcattgacaa tgtctccttg gctaaggcct gcacaatatt taattatatc
13561 ccaaaatgag tggccaataa attcacgtgg aaaatctgat cgcgcagaga taaaaaggaa
13621 gttttatgga gtgtaagcag aaaggctcatc aagttatagt tgaagaaatt aaatatatgt
13681 tgaaagaaat ggacgtcagg atggatgaca attttaccga tttaggaggg aactcaatca
13741 tggtatgat aattacggat aatttgcaga aaaaatattc tattaatata gaggtagccc
13801 aactgctggg tagtaagatt ggtgaaattg aactgaaacc tctgggcaag taaatgaaaa
13861 tattaagtac agggcataca ttatcgcgag ccgcacatct tgacgaagtt gatcatccta
13921 atcagttaca gctagaagcg atcaaacgtg cgggttataa gcaattcttg actgaatctc
13981 agagcgttcg ggaaatggct gcctcagctg cagaaaatgc actggaacaa ttccatttat
14041 cttccgaaga tgtaggggtt attgtcgctg gggtttcggg agttcccgat tttattggta
14101 tcgacttagc ctgtcagggt ggagcagaac taaattgtaa tcagattcgt aactcaact
14161 tagttgaggg ttgtgctacg gctgtcagtg tttggaagca tgcattctca ctttgtactg
14221 agatgccaga aggtaaattt gggctggtag tactggcgca gcgcattgag gatactcatc
14281 aggacagatt cggacttatg aatgctgcgt taagtgatgg agcagttgct tgcgtggtag
14341 gaaaggcaat cacatatgca gatcagcccg gattgcgtta tatacttaca gaagatattt
14401 ctgactgccg ttatgtagat atgatgcgca tagaatatgg tgggtggacat ttacctttcc
14461 tacctgaagg aagagatagc aaaaaagata aattaggtcg agagcgaatt atggataatt
14521 attgtttctc atctcaggat ttaatgaatt ttttaatttt acgcgaaaat aacagtatca
14581 atgtcattcg tcgtgtattg gagaaatctg taagcctaga tactcctcct tttctactac
14641 atactctcga aggaaaacag agcatagaaa atttatgtaa ccgaattggg attccgattg
14701 agagaagtaa tattagcttg ttatcggagt taggacatgt aggttcgccc gaccgcgtgt
14761 tgtcatttcg attgatgatg cagcgtggga taatcagtcg tggttcagag attgtcatga
14821 gtactatttc taccggcatg aaatggggag catccctttt ccggtatgaa accgctataa
14881 gtaaggataa tgtaagctat aaaccagaaa ggagtaataa ttaa

A2.2. Protein sequence similarities of the translated DNA sequence located upstream of the AGA gene cluster

Table A2. Similarities to the region upstream of the AGA gene cluster

Code	Accession no.	Putative functions; origin	size	score	(bits)
PCR	(AF336309)	probable transposase gene; a low calcium response plasmid from <i>Yersinia enterocolitica</i> Serotype 0:8	244	8e-59	(230)
PB1	(NC_002142)	resolvase (RsvB); adherence factor plasmid pB171 from <i>Escherichia coli</i>	269	4e-42	(174)
PCC	(P18021)	resolvase; plasmid pColBM-C1139 from <i>Escherichia coli</i>	260	6e-42	(174)
PCP	(NC_002122)	resolvase; plasmid pColIb-P9 from <i>Shigella sonnei</i>	259	8e-42	(173)
PF	(NC_002483)	resolvase; plasmid F from <i>Escherichia coli</i> K-12	268	2e-41	(172)
PV	(NC_002638)	Rsd; a 50k virulence plasmid from <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Choleraesuis</i>	260	3e-41	(171)

Blast X alignment of the translated DNA sequence upstream of the AGA gene cluster from Eh1087 (EH) with other protein sequences in the GenBank / EMBL / XXX databases:

Figure A2. Sequence alignments upstream of the AGA gene cluster

EH	1916	LGSLPRELTQISRSVDVTGQNVTSGGAARYFDYESAVALRNLAAYGTDMPRYLLAPEVAVLLSKVPDLKKRLFDITLWNTGGR
PCR	2	L +P E +QISRS +T YF+YE AVALR +A G +PRYLLAPE+ VLL +PDL++R L DTLWNTGR
		L SLIPHENSQISRSAITP-----YFNYEKAVALRRMALKAGASLPRYLLAPEITVLLDYL PDLRQRLLFDTLWNTGAR
PB1	31	DY +A+ALR ++ + ++P+YLLAPEV+ LL VPDL++++ + TLWNTG R
		DYPAALALRQMSMVHD-ELPKYLLAPEVSALLHYVPDLRRKMMLLATLWNTGAR
PCC	22	DY +A+ALR ++ + ++P+YLLAPEV+ LL VPDL++++ + TLWNTG R
		DYPAALALRQMSMVHD-ELPKYLLAPEVSALLHYVPDLRRKMMLLATLWNTGAR
PCB	22	DY +A+ALR ++ + ++P+YLLAPEV+ LL VPDL++++ + TLWNTG R
		DYPAALALRQMSMVHD-ELPKYLLAPEVSALLHYVPDLRRKMMLLATLWNTGAR
PF	31	DY +A+ALR ++ + ++P+YLLAPEV+ LL VPDL +++ + TLWNTG R
		DYPAALALRQMSMVHD-ELPKYLLAPEVSALLHYVPDLHRKMMLLATLWNTGAR
PV	23	DY +A+ALR +A + D P+YLLAPEV+ LL VPDL +R+ + TLWNTG R
		DYPAALALRQMAMQHD-DYPKYLLAPEVSALLHYVPDLHRRMLLATLWNTGAR
EH		LNEILPLTGGGFCAGRSAHRRPAEFTVRGAHPQAAEAGRGGPQPPARASDKXXXXXXXXXXGDPGEPPPRAVPLTDPGFVQ
		+NE L LT P +F + GA + + + P R + GE R VPL+DP +V+
PCR		INECLVLT-----PGDFELDGAGPFVTLTKLQHRGPGRPEN-----GEALKRIVPLSDPAYVR
		+NE L LT G F P F + +A R + PA G+ R VPL+D +V
PB1		INEALALTRGDFSLAP-----PYPFVQLATLKQRTAKAARTAGRTPA-----GQQTTRLVPLSDSWYVS
		+NE L LT G F P F + +A R + PA G+ R VPL+D +V
PCC		INEALALTRGDFSL-----TPPYPFVQLATLKQRTAKAARTAGRMPPA-----GQQTTRLVPLSDSWYVS
		+NE L LT G F P F + +A R + PA G+ R VPL+D +V
PCB		INEALALTRGDFSL-----TPPYPFVQLATLKQRTAKAARTAGRMPPA-----GQQTTRLVPLSDAWYVS
		+NE L LT G F P F + +A R + PA G+ R VPL+D +V
PF		INEALALTRGDFSLAP-----PYPFVQLATLKQRTAKAARTAGRMPPA-----GQQTTRLVPLSDSWYVS
		+NE L LT G F P F +A +A R + P+ G P R VPL+D +V
PV		INEALALTRGDFSLAP-----PYPFVQLATLKQRAEKAARTAGRMPS-----GSQPHRLVPLSDNQYVS
EH		RLREWFATAQLPAGSRL-----WDIRSEDRTARSWISQAVASAAARDGVTFISIRPVTPTKTFRDSFAMHLVQHQVQPKVI
		RL+E+ AT ++ L W ++S+DTAR+W+ AV A RDGVTF+I P+TP TFR SFAMHL+Q+ +P KV
PCR		RLQEYAATMKITRRKPL-----WPKSDDTARNWLKAAVQRAERDGVTFITPITPHTFRHSFAMHLLQNLGPFKVL
		+L+ AT++ P +R+-----W++ ++ T R+WI +AVA+AA DGVTF+ PVTPTFRS+AMH++ +P KV+
PB1		QLQTMVATLKIPLERRNRKRTGRTEKWEV-TDRTVRTWIGEAVAAAATDGVTF+SV-PVTPTFRHSYAMHMLYAGIPLKVL
		+L+ AT ++P +R+ W++ ++ T R+WI +AVA+AA DGVTF+ PVTPTFRS+AMH++ +P KV+
PCC		QLQTMVATLKIPMERRNRKRTGRTEKARIWEV-TDRTVRTWIGEAVAAAADGVTF+SV-PVTPTFRHSYAMHMLYAGIPLKVL
		+L+ AT ++P +R+W++ ++ T R+WI +AVA+AA DGVTF+ PVTPTFRS+AMH++ +P KV+
PCB		QLQTMVATLKIPMERRNRKRTGRTEKARIWEV-TDRTVRTWIGEAVAAAADGVTF+SV-PVTPTFRHSYAMHMLYAGIPLKVL
		+L+ AT ++P +R+W++ ++ T R+WI +A VA+AADGVTF+ PVTPTFRS+AMH++ +P KV+
PF		QLQTMVATLKIPMERRNRKRTGRTEKARIWEV-TDRTVRTWIGEAVAAAADGVTF+SV-PVTPTFRHSYAMHMLYAGIPLKVL
		L+ AT ++P +RLW+I ++ T R+WI +AV +AA D VTF+ PVTPTFRS+AMH++ +P KV+
PV		ELQMMVATLKIPLERRNRKRTGRTEKARLWEI-TDRTVRTWIGEAVEAAAADGVTF+SV-PVTPTFRHSYAMHMLYAGIPLKVL
EH		KTLMGHKDAKSTEWYTRVFALDVTRQLGVRFMTDPQAEAGRLLLPR 1068
		++ MGH+D KSTE YTR+VFALDV Q GVRF+MD +A LLLP
PCR		QAYMGHQDTKSTEIYTRIFALDVGHQYGVRFSDMTLDARALLP 244
		++LMGHK STE YT+VFALDV + V+F+M +A +L R
PB1		QSLMGHKSSSTEYVTKVFALDVAARHRVQFSMPESDAVTLKKNR 267
		++LMGHK STE YT+VFALDV + V+F+M +A +L R
PCC		QSLMGHKSSSTEYVTKVFALDVAARHRVQFSMPESDAVTLKKNR 258
		++LMGHK STE YT+VFALDV + V+F+M +A +L
PCB		QSLMGHKSSSTEYVTKVFALDVAARHRVQFSMPESDAVSML 255
		++LMGHK STE YT+VFALDV + V+F M +A +L
PF		QSLMGHKSSSTEYVTKVFALDVAARHRVQFAMPESDAVAML 264
		+ LMGHK STE YT+VFALDV + V+F M +A +L
PV		QALMGHKSVSSTEYVTKVFALDVAARHRVQFQMPGADAVAML 256

A2.3. Protein sequence similarities of the translated DNA sequence data located downstream of the AGA gene cluster

Table A2. Similarities to the region downstream of the AGA gene cluster

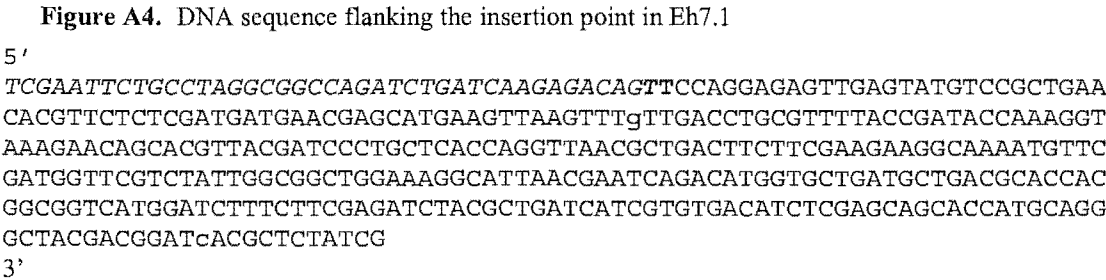
Code	Accession no.	Putative functions; origin	size	score	(bits)
RR	(NC_002575)	probable transposase gene; root-inducing plasmid pRi1724 from <i>Rhizobium rhizogenes</i>	475	1e ⁻¹⁰	(47)
AT	(AF065242)	unknown; soybean-supervirulent chrysopine-type Ti plasmid pTiChry5 from <i>Agrobacterium tumefaciens</i>	464	7e ⁻⁰⁹	(45)
RN	(NC_000914)	putative transposase Y4BF; plasmid pNGR234a from <i>Rhizobium</i> sp. NGR234	457	5e ⁻⁰⁷	(39)
ST	(NC_002305)	putative transposase; IncHI plasmid R27(heat- sensitive transfer) from <i>Salmonella typhi</i>	468	2e ⁻⁰⁶	(37)
SE	(AL513383)	putative transposase; plasmid pHCM1 from <i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhi</i> strain CT18	465	1e ⁻⁰⁴	(34)

Blast X alignment of the translated DNA sequence downstream of the AGA gene cluster from Eh1087 (EH) with other protein sequences in the GenBank / EMBL / XXX databases.

Figure A3. Sequence alignments downstream of the AGA gene cluster

EH	13	INARGRLPGTHERIDETLRYRILTLLDEYYASFSGSISTCHRKLLERYEINISTETQRCWMTTYGLWNLHICHQTHIYHPR*NHP	261
		++ +R P R E R L L+ E Y FG + KL+E + I+++ ET R WMT G+W + ++ PR	
RR	58	VSKKRLQPSNRRHTEEFRNAALDLIRERYLDFGP-TLAREKLIELHRISVAKETLRQWMTEAGIWSRRERKKRVFQPR	135
		G P +RI +R +TL+ E YA FG + KL ER + +S ET R WMT G W	
AT	68	GRPSNNRISAGVRDYAMTLVRERYADFGP-TLATEKLAERNGLRVSRETVRSWMTDAGPW	126
		G P +RI + +R + ++ E YA FG + KL E + +S ET R WM GLW	
RN	62	GRPSNNRICDGVRDYAMAIVRERYADFGP-TLAAEKLAELDGLTVSRETLRQWMADAGLW	120
		RRG +++ + L L ++ E Y FG C KL E + ++IS ET R MT	
ST	63	RRGKSSNNQLPQGLAAYALNIIRERYNDFGPTLACE-KLAEVHGVHISKETVRKLMTQASLW	123
		N RG G + +L + L+++ E Y FG + KL E + + + ET R M GLW I+ PR P	
SE	61	NQSRGRTGNRLLPSTLTDQALSIIRERYRDFGP-TLAREKLEEVHGLVLGKETIRRLMIKAGLWIPRRQAPKIHQPYYRRP	141
EH	248	GEIIQIIGAHVWFEARTSKYSLLIHVDD	334
		GE++QI G+HH WFE R K +LL+++DD	
RR	142	GELVQIDGSHHWWFENRGPKCALVYIDD	170
		GE++QI G+ H WFE R SLL+ VDD	
AT	146	GELVQIDGSEHRWFEDRGPCSLLVFVDD	174
		GE++QI G+ H WFE R SLL+ +DD	
RN	140	GELVQIDGSEHRWFEDRGDPCSLLVFIDD	168
		GE+IQI G H WFE R K + L++VDD	
ST	144	GELIQIDGCDHHWFENRGPKCTALVYVDD	172
		GE+IQI G H WFE R + L++VDD	
SE	144	GELIQIDGCDHHWFENRGPCALVYVDD	172

A2.4. Sequence similarity data for Eh7.1



DNA sequence using primer T3, which anneals to the vector pBluescriptKS-. The sequence in italics is mini-Tn5*lacZ2*, and the transposon insertion point is highlighted in bold.

Table A3. BlastN alignments of DNA sequence flanking the Eh7.1 insertion

Sequences producing significant alignments:		Score (bits)	E Value
gi 41562 emb X05173.1 ECGLN	<i>E. coli glnALG</i> operon with <i>glnA</i> ...	232	2e-58
gi 146156 gb M13746.1 ECOGLNAB	<i>E. coli glnA</i> gene encoding <i>glnA</i> ...	228	3e-57
gi 154089 gb M14536.1 STYGLNA	<i>S. typhimurium glnA</i> gene ...	214	5e-53
gi 3808289 gb AF072440.1 AF072440	<i>Enterobacter gergoviae glnA</i> ...	188	3e-45
gi 155170 gb L08499.1 VIBGLNNTR	<i>Vibrio alginolyticus</i> glutamine...	153	1e-34
gi 146158 gb J01618.1 ECOGLNACR	<i>Escherichia coli</i> K-12 glutamine...	131	5e-28

Glutamine but not glutamate was found to restore growth and AGA-production to strain Eh7.1 grown in minimal media. Taken with the the sequence similarities presented here it is concluded that Eh7.1 possesses a mini-Tn5*lacZ2* insertion in the Eh1087 *glnA* gene, the product of which is responsible for conversion of glutamate into glutamine. It was further demonstrated that the transposon insertion is solely responsible for glutamine auxotrophy by transferring the transposon insertion in Eh7.1, via an Eh1087 *glnA*-encoding cosmid, to Eh1087, recreating a glutamine auxotroph.

A2.5. Sequence similarity data for Eh56F2

Figure A5. DNA sequence flanking the insertion point in Eh56F2

5'
 CTCCGATAGCACGAAGGCCGGGATAATGAGCNGCATATTGTCGGGGGTGACCGAATCTGCCATTTTCTT
 TGGCCACATACGCTGGGCATTTTCTGAGTTCGAAGAGGTCATTACTCAGGTCGCTCGTGACGGCACG
 CCACACGTGATGTGTGCCATCTCTACGATcTgGCCGGTCTGTTCTCCGATTCTACGAGCACTGCCCG
 ATTCTCACCGCAGAATCAGACACAACCCGTCTGAGCCGACTGCAGTTCATTGTAACGGTGCACCACAAA
 AAATGAGCTGAGCAGCAGGCCAACCATcAGCGTGGGTGCCAGAATTAAAATCATCATTCGCGcCGCAG
 GCTGTATTTGGTCATAATAGTCGCCTGGCTCTGTCTCTTGATCAGATCTGGCCGCCTAGGCGAATTCCC
 3'

DNA sequence using primer T7, which anneals to the vector, pBluescriptKS-. The transposon insertion point is highlighted in bold (between bases 375 and 376), and the sequence in italics is from mini-Tn5*lacZ*. A start codon (reverse-complement) for a potential *E. coli barA* homolog is underlined.

Figure A6. Alignments of the sequence flanking the Eh56F2 insertion point

A) Translated sequence alignment

Translated protein sequence similarity: *E. coli* Sensor protein BarA (Swiss prot: P26607)

Score = 70.6 bits (170) Expect = $6e^{-12}$

Identities = 54/120 (45%); for first 38 bases: 32/38 (84%)

Positives = 74/120 (61%); for first 38 bases: 36/38 (95%)

```

Eh56F2: 360 MTKYSLRARMILILAPTLMVGLLLSSFFVHRYNELQSAQTGCV*FCGENRAVLVESG- 184
          MT YSLRARMILILAPT+++GLLLS FFVHRYN+LQ           E AV E G
BarA:    1   MTNYSLRARMILILAPTVLIGLLLSIFFVHRYNDLQRQLEDAGASIIIEPLAVSTEYGM 60

Eh56F2: 183 -----EQTGQIVEI---GTHHVWRA-----VTS DLSNDLFELQENARMWPKKM 61
          E  GQ++ +      + RA          VTS+ D  +Q +  +P+++
BarA:    61  SLQNRESIGQLISVLHRRHSDIVRAISVYDENNRFLVTSNFDLPSSMQLGSNVFPFRQL 120

Eh56F2: 60  ADSVTPDNMXLIIPAFVLSE 1
          +VT D  +I+  ++SE
BarA:    121 --TVTRDGDIMILRTPIISE 138

```

B) DNA sequence alignment

DNA sequence similarity: *Escherichia coli argS* gene for arginyl-tRNA-synthetase (X15320.1)

Score = 103 bits (52); Expect = $5e^{-20}$; Identities = 85/96 (88%)

```

Eh56F2: 127  cgtgacggcacgccacacgtgatgtgtgcctatctctacgatctggccgggtctgttct 184
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
argS:    1782 cgtgaaggcacgccgatgtaatgtgtgtcttacctgtacgatctggccgggtctgttct 1839

Eh56F2: 185  ccggattctacgagcactgcccgattctcaccgcagaa 222
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
argS:    1840 ctggcttctacgagcactgcccgatcctcagcgagaa 1877

```

A2.6. The Eh1087 *recA* gene

Figure A7. DNA sequence of the Eh1087 *recA* gene

5'
 CTGCAGAACTGTATGGCGAATTCTCTCGACAAATTAACCTTGATACTGTATGACTATACAGTATAATT
 GTCGGCAACAGANAGAGTACAGGCACCCTGAGTGCGTGTTTACCCGGCTTGATTAGGAGTTGAA**ATGGC**
 GATTGATGAAAACAAACAGAAGGCTTTAGCTGCCGCGCTTGGCCAGATTGAGAAGCAATTTGGTAAAGG
 CTCCATCATGCGCTTGGGTGAAGACCGCTCAATGGATGTGGAAACTATCTCTACCGGCTCGCTGTCGCT
 GGATATCGCATTAGGCGCAGGTGGTCTGCCAATGGGCCGTATCGTTGAGATCTACGGACCTGAGTCTTC
 AGGTAAACACGCTGACTTTGACAGGTTATCGCATCTGCACAGCGTAAAGGTAAACCTGTGCCTTTAT
 CGATGCAGAACATGCGCTTGATCCGGTTTACGCCAAGAACTCGGCGTAGACATTGATAACCTGCTCTG
 CTCTCAGCCAGACACTGGTGAGCAGGCGCTGGAAATCTGTGATGCGCTGGCGCGCTCTGGTGCCGTTGA
 CGTGATCATCGTCGACTCCGTGCGGCGCTGACGCCTAAAGCGGAAATCGAAGGTGAAATCGGTGACTC
 ACACATGGGCCTCGCGGCACGTATGATGAGCCAGGCGATGCGTAAACTGGCCGGTAACCTGAAGCAGTC
 CAATACCCTGCTGATTTTCATCAACCAGATCCGTATGAAAATTGGTGTGATGTTTGGTAACCCGGAAC
 CACCCTGGTGGTAAACGCGCTGAAGTTCTACGCCTCTGTCCGTCTTGATATCCGCCGTATCGGTGCAAT
 CAAAGAGGGCGATAACGTGGTCCGCGAGTGAGACCCGCGTTAAAGTGGTGAAAAACAAATCGCCGCGCC
 ATTCAAACAGGCTGAATTCCAGATCATGTACGGCGAAGGTATCAATACCTTTGGTGAGCTGGTTCGACCT
 GGGCGTGAAGCACAACTGATTGAAAAAGCGGGTGCATGGTACAGCTACAATGGCGACAAGATTGGTCA
 GGGTAAATCGAATGCCGGCAACTTCCTGAAAGAGAATGCTGCTGTGGCAAACGAGATTGATCTGAAGTT
 ACGTGAGATGTTGCTCAACGGCGCGGATGAAAACTCGCAGCAGCCGACAAGGCTGCTGAGAAAGAAGA
 CGCGGCTGAAGCGAACGAAGACTTCTAATCTCT**GTATG**CGGGAGGGGCGAAAGCCTCTCCCGTAGTTTTTA
 TCCCCCTCTATTATCTTTCTTGCCCTTCCCTCTGTCTCTGTTCTGTCTGCCATAATCTCCCTCTTTTTGTAC
 AAGGTGCGCAGTATGACTGAACCCCTCTCAGCCCCAACCTAATGTCTGTCTCTTACTCTCGTCTGCTTGAC
 CGTGCGATGCGTATTCTGGGTCAAGCGGATCACAGCCGTGCAGAGTTAGCGCGTAAACTGCAACAATCA
 GCGCAGCGTGCTGCGTGGGCGCAGAAAAAAGAGCCCGAAATTGTTACCGAAGCGTTGCTGGAACAGGTT
 CTAACTGGTGTGAGGAAAGCGGCTGGTTAAACGACGAGCGTTTCACCGAAGCATTATTAGAGCCGG
 AGCCGTAAGGGCTTTGGATCGCAGCGCGTACGCCTTGAACTGGCCAGAAAGGCATTGACCGTGAAGCC
 ATTGATCTTGCAATGGHAGAGACAGAGGTAGACTGGGCGGCGTGTGCGGYTCAGCTGGCTGAACGAAAA
 TTCGACACCCGCTGCCACAGAGTGAAAGNGAAAGCGAAAGTACTACGCTATCTGCAG
 3'

DNA sequence of a 1784 bp DNA fragment, bordered by *Pst*I sites (italics), that encodes the Eh1087 *recA* gene. Predicted codons for translation initiation (ATG) and termination (TGA) of the 1068 bp *recA* gene (respectively) are shown in bold. An Ω Cm^R cassette used to create *recA* mutants was inserted in the underlined *Eco*RI site.

A3. Data summary for all AGA gene cluster mini-Tn5*lacZ2* insertion mutants

Table A4. Insertion point data for all Eh1087 AGA-minus mutants

Orientation 1 insertions						Orientation 2 insertions					
No.	Strain	Position ¹	β -gal ²	<i>ehp</i> ³	colour ⁴	No.	Strain	Position	β -gal	<i>ehp</i>	colour
1	S2.1	1600	(+)	A	pale	1	M97.1	1700	(-)	A	pale
2	M14.1	1800	(-)	A	pale	2	M104.2	2300	(-)	B	pale
3	M14.5	2000	(+)	B	pale	3	M54.1	2800	(-)	C	pale
4	M14.4	2800	(-)	C	pale	4	M80.1	3100	(-)	C	pale
5	M26.1	2850	(-)	C	pale	5	M72.1	3200	(-)	C	pale
6	M102.1	2900	(-)	C	pale	6	M101.1	3400	(-)	C	pale
7	S12.2	3000	(-)	C	pale	7	S13.3	3500	(-)	C	pale
8	S5.1	3200	(-)	C	pale	8	M62.3	3600	(-)	C	pale
9	M34.1	3300	(-)	C	pale	9	M24.2	3700	(-)	C	pale
10	M67.3	3400	(-)	C	pale	10	M86.1	3750	(-)	C	pale
11	S13.2	3600	(+)	C	pale	11	M24.1	4000	(-)	C	pale
12	M62.1	3800	(+)	C	pale	12	M103.1	4200	(-)	D	pale
13	S14.1	4100	(-)	C	pale	13	M3.2	4800	(-)	D	pale
14	M24.3	4400	(-)	C	pale	14	M97.2	6900	(-)	F	yellow
15	M67.1	4800	(-)	D	pale	15	M35.2	7800	(-)	G	yellow
16	M49.1	4900	(+)	D	pale	16	S1.1	8350	(-)	H	yellow
17	M53.1	4900	(+)	D	pale	17	M104.1	8450	(-)	H	yellow
18	S3.4	5200	(-)	D	pale	18	M62.2	8800	(-)	I	red
19	M3.1	5800	(-)	E	light yellow	19	M4.1	8800	(-)	I	red
20	M70.1	5900	(-)	E	light yellow	20	M31.1	9000	(-)	I	red
21	M103.2	6150	(+)	F	yellow	21	M55.1	9400	(-)	J	red
22	S12.4	6300	(+)	F	yellow	22	M101F1	9500	(-)	J	red
23	M28.1	6900	(+)	F	yellow	23	S11.1	9600	(-)	J	red
24	M88.1	7100	(-)	G	yellow	24	S10.1	10000	(-)	J	red
25	M21.3	7500	(+)	G	yellow	25	M98.1	11200	(-)	K	red
26	M53.2	7900	(-)	G	yellow	26	M104F1	11500	(-)	L	red
27	M61.1	8000	(-)	G	yellow	27	M18.2	11500	(-)	L	red
28	M21.1	8200	(-)	H	yellow	28	S3.1	12700	(-)	M	orange
29	M14.2	8400	(-)	H	yellow	29	M56.1	12950	(-)	M	orange
30	M6.1	8800	(-)	I	red	30	M67.2	13100	(-)	M	orange
31	M4.2	8800	(-)	I	red	31	M13.2	13600	(-)	M	orange
32	M65.1	8800	(-)	I	red	32	S13.1	14200	(-)	N	orange
33	M30.1	8850	(-)	I	red	33	M63.1	14200	(-)	N	orange
34	M87.1	8900	(+)	I	red	34	M35.1	14500	(-)	O	orange
35	M13.4	9100	(+)	I	red						
36	M23.1	9200	(-)	I	red						
37	M14.3	9500	(-)	J	red						
38	M52.1	10000	(-)	J	red						
39	M20.2	10500	(-)	J	red						
40	M66.1	10900	(-)	K	red						
41	M49.2	12300	(+)	L	brown						
42	M68.1	13200	(+)	M	orange						
43	M26F1	13300	(+)	M	orange						
44	M103.3	13700	(-)	M	orange						
45	S10.2	13800	(+)	M	orange						
46	S4.2	14700	(-)	O	orange						
47	M93.1	14900	(-)	O	orange						

Key:

1. Number of bases between a *Bam*HI site upstream of *ehpR* and each transposon insertion
2. β -galactosidase activity resulting from in-frame active fusions between *lacZ* and an *ehp* gene
3. The *ehp* gene interrupted in each mutant
4. The colour of compounds released by each mutant grown in minimal or Kings A media

A4. NMR data for D-alanylgriseoluteic acid.

Figure A8. The chemical structure of AGA with NMR data

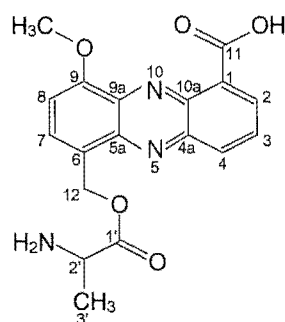


Table A5. ^{13}C NMR data and long range ^1H - ^{13}C correlations (CIGAR) for AGA

Atom number	$\delta^1\text{H}$ (ppm, mult, J Hz)	$\delta^{13}\text{C}$ ppm	Correlated carbons
1	-	124.9	-
2	8.95 (d, 7.5)	136.8	C-4, C-10a, C-11
3	8.05 (dd, 7.5, 1.5)	130.6	C-1, C-4a
4	8.51 (d, 8)	134.6	C-2, C-10a
4a	-	142.7	-
5a	-	142.7	-
6	-	126.1	-
7	7.92 (d, 8)	131.5	C-5a, C-9, C-12
8	7.16 (d, 7.5)	107.6	C-6, C-9, C-9a
9	-	154.0	-
9a	-	132.9	-
10a	-	138.5	-
11	-	165.7	-
12	5.9 (d, 12.5), 5.85 (d, 12.5)	62.0	C-5a, C-6, C-7, C-1'
1'	-	176.4	-
2'	3.64 (m)	49.9	-
3'	1.36 (d, 6.5)	20.6	C-1', C-2'
9-OCH ₃	4.18 (s)	56.4	C-9

¹Data determined and provided by Yunjiang Feng, Chemistry Dept., Canterbury University, New Zealand.

A5. Blossom assay data

Table A6. Blossom assay data

Assay A) Populations on stigma first inoculated with Eh1087 or EhΔAGA

0 h	Ea8862	Eh1087	EhΔAGA	Ea8862 x Eh1087		Ea8862 x EhΔAGA	
	Nal	Rf	Rf + Sm	Nal	Rf	Nal	Rf + Sm
1	1.6E+04	2.1E+06	9.3E+05	2.8E+03	3.0E+06	2.8E+03	1.1E+07
2	1.3E+04	9.6E+05	7.8E+05	7.5E+03	1.1E+07	2.9E+03	2.3E+06
3	8.3E+03	1.6E+06	7.6E+05	3.1E+03	5.2E+06	3.8E+03	2.8E+06
4	1.3E+04	1.3E+06	5.1E+05	1.3E+03	7.8E+06	5.2E+03	6.8E+06
5	2.3E+04	1.9E+06	3.6E+05	7.9E+03	4.2E+06	5.2E+03	3.2E+06
6	2.1E+04	6.3E+05	8.3E+05	2.6E+03	6.2E+06	6.8E+03	1.9E+06
7	2.0E+04	4.1E+05	2.7E+05	2.6E+03	3.7E+06	4.0E+03	1.5E+06
8	1.5E+04	2.0E+06	4.5E+05	4.4E+03	1.0E+07	3.5E+03	4.1E+06
Av	1.6E+04	1.3E+06	6.1E+05	4.0E+03	6.4E+06	4.3E+03	4.2E+06
SD	4.9E+03	6.4E+05	2.4E+05	2.4E+03	3.0E+06	1.4E+03	3.2E+06

12 h	Ea8862	Eh1087	EhΔAGA	Ea8862 x Eh1087		Ea8862 x EhΔAGA	
	Nal	Rf	Rf + Sm	Nal	Rf	Nal	Rf + Sm
1	1.9E+05	1.7E+06	1.9E+06	1.8E+05	8.1E+06	3.5E+03	1.1E+07
2	3.2E+05	1.7E+06	1.2E+06	5.4E+03	4.1E+06	9.9E+03	4.3E+06
3	1.5E+05	2.3E+06	1.5E+06	2.4E+03	9.9E+06	5.0E+04	1.9E+07
4	5.4E+04	1.9E+06	1.9E+06	1.6E+04	7.7E+06	4.8E+04	8.5E+06
5	3.7E+05	1.8E+06	9.3E+05	1.0E+05	1.3E+07	5.5E+04	8.8E+06
6	4.7E+05	2.7E+06	2.1E+06	7.0E+02	1.7E+07	6.8E+04	2.6E+06
7	8.6E+04	4.6E+06	1.1E+06	3.1E+04	1.4E+07	1.1E+04	3.2E+06
8	1.4E+05	1.1E+06	2.2E+06	4.2E+03	5.7E+06	8.0E+03	2.2E+07
Av	2.2E+05	2.2E+06	1.6E+06	4.2E+04	1.0E+07	3.2E+04	9.9E+06
SD	1.5E+05	1.1E+06	4.8E+05	6.5E+04	4.4E+06	2.6E+04	7.1E+06

24 h	Ea8862	Eh1087	EhΔAGA	Ea8862 x Eh1087		Ea8862 x EhΔAGA	
	Nal	Rf	Rf + Sm	Nal	Rf	Nal	Rf + Sm
1	2.5E+06	4.6E+05	8.7E+06	6.9E+02	3.2E+07	8.8E+04	5.4E+06
2	4.6E+06	2.9E+05	2.9E+06	7.3E+02	1.0E+07	3.6E+03	7.9E+06
3	1.2E+07	2.2E+05	6.0E+06	3.2E+02	1.7E+07	2.7E+05	4.1E+06
4	6.1E+06	3.8E+05	3.5E+06	1.4E+04	1.8E+07	6.0E+04	2.1E+07
5	8.6E+06	1.5E+06	4.5E+06	3.1E+02	3.1E+07	4.7E+04	1.2E+07
6	4.3E+06	2.0E+06	2.6E+06	1.7E+04	2.7E+07	6.5E+04	6.7E+06
7	1.8E+07	2.5E+06	2.9E+06	1.1E+03	2.4E+07	1.0E+04	7.8E+06
8	9.4E+06	4.0E+06	3.8E+06	1.1E+04	1.1E+07	4.5E+04	5.3E+06
Av	8.2E+06	1.4E+06	4.4E+06	5.6E+03	2.1E+07	7.4E+04	8.7E+06
SD	5.0E+06	1.4E+06	2.1E+06	7.1E+03	8.6E+06	8.4E+04	5.3E+06

48 h	Ea8862	Eh1087	EhΔAGA	Ea8862 x Eh1087		Ea8862 x EhΔAGA	
	Nal	Rf	Rf + Sm	Nal	Rf	Nal	Rf + Sm
1	5.6E+07	1.1E+07	7.0E+06	2.4E+03	1.4E+07	1.2E+05	9.5E+06
2	7.8E+07	1.0E+07	8.0E+06	2.2E+04	1.4E+07	4.5E+07	7.2E+06
3	1.7E+07	3.6E+06	6.7E+06	4.9E+03	1.3E+07	2.6E+05	1.8E+07
4	6.3E+07	8.0E+06	1.1E+07	9.6E+05	2.9E+07	1.9E+07	1.6E+07
5	5.7E+07	5.0E+06	7.7E+06	8.8E+04	2.4E+07	2.7E+05	5.1E+06
6	3.6E+07	7.3E+06	4.7E+06	5.1E+04	9.2E+06	1.0E+06	3.2E+07
7	8.6E+07	3.9E+06	3.6E+06	2.1E+04	2.7E+07	1.7E+06	1.6E+07
8	4.3E+07	4.8E+06	4.5E+06	6.4E+03	1.0E+07	6.3E+05	8.6E+06
Av	5.5E+07	6.7E+06	6.7E+06	1.4E+05	1.8E+07	8.5E+06	1.4E+07
SD	2.2E+07	2.8E+06	2.4E+06	3.3E+05	7.9E+06	1.6E+07	8.6E+06

72 h	Ea8862	Eh1087	EhΔAGA	Ea8862 x Eh1087		Ea8862 x EhΔAGA	
	Nal	Rf	Rf + Sm	Nal	Rf	Nal	Rf + Sm
1	1.9E+08	1.0E+07	1.1E+07	9.0E+04	2.8E+07	7.0E+05	1.9E+07
2	7.3E+07	1.3E+07	1.7E+07	1.5E+05	2.4E+07	1.9E+05	2.8E+07
3	8.0E+07	5.6E+06	7.2E+06	2.1E+02	2.3E+07	2.1E+05	3.9E+07
4	5.6E+08	5.7E+06	2.0E+07	1.2E+04	2.0E+07	5.3E+05	1.7E+07
5	5.3E+07	8.4E+06	8.2E+06	9.7E+03	2.2E+07	8.7E+04	1.5E+07
6	6.1E+07	1.2E+07	1.7E+07	3.5E+03	8.9E+06	4.2E+05	2.4E+07
7	7.4E+07	1.6E+07	1.6E+07	1.4E+04	3.9E+07	2.4E+05	1.5E+07
8	1.3E+08	5.9E+06	1.7E+07	7.4E+02	1.3E+07	9.0E+05	3.7E+07
Av	1.5E+08	9.5E+06	1.4E+07	3.5E+04	2.2E+07	4.1E+05	2.4E+07
SD	1.7E+08	3.8E+06	4.7E+06	5.5E+04	9.2E+06	2.8E+05	9.7E+06

Assay B) Populations on stigma inoculated with strains simultaneously

12 h Ea8862 x Eh1087			Ea8862 x EhΔAGA			24 h Ea8862 x Eh1087			Ea8862 x EhΔAGA		
	N	R	N	R	R + S		N	R	N	R	R + S
1	3.5E+04	3.5E+05	8.6E+04	8.0E+05	7.4E+05	1	9.9E+04	1.7E+06	1.9E+06	2.7E+06	3.9E+06
2	1.1E+04	1.8E+05	1.4E+05	3.2E+05	4.6E+05	2	1.4E+05	5.3E+06	3.4E+06	4.0E+06	3.4E+06
3	1.5E+04	2.2E+05	5.8E+04	5.3E+05	5.0E+05	3	1.1E+05	3.7E+06	7.0E+06	6.9E+06	6.4E+06
4	2.3E+04	2.6E+05	3.5E+04	5.2E+05	4.1E+05	4	6.7E+04	3.1E+06	4.4E+06	3.6E+06	2.7E+06
5	6.9E+03	7.3E+04	5.4E+04	4.0E+05	4.2E+05	5	1.1E+05	1.7E+06	8.2E+06	5.4E+06	3.7E+06
6	5.4E+04	3.7E+05	2.7E+04	3.8E+05	2.3E+05	6	3.7E+05	1.5E+06	7.1E+06	6.9E+06	6.2E+06
7	1.9E+04	2.0E+05	7.0E+04	1.0E+06	8.0E+05	7	1.5E+05	2.1E+06	2.7E+06	3.1E+06	2.6E+06
8	2.7E+04	3.2E+05	5.8E+04	3.6E+05	3.0E+05	8	7.4E+05	4.2E+06	3.4E+06	1.1E+07	3.6E+06
Av	2.4E+04	2.5E+05	6.6E+04	5.4E+05	4.8E+05	Av	2.2E+05	2.9E+06	4.8E+06	5.4E+06	4.1E+06
SD	1.5E+04	9.9E+04	3.5E+04	2.5E+05	2.0E+05	SD	2.3E+05	1.4E+06	2.3E+06	2.7E+06	1.5E+06

48 h Ea8862 x Eh1087			Ea8862 x EhΔAGA			72 h Ea8862 x Eh1087			Ea8862 x EhΔAGA		
	N	R	N	R	R + S		N	R	N	R	R + S
1	7.3E+04	1.1E+07	1.8E+07	4.8E+06	3.3E+06	1	5.5E+05	4.1E+06	2.1E+07	4.7E+06	6.1E+06
2	2.6E+06	5.2E+06	1.8E+07	4.4E+06	5.8E+06	2	6.4E+07	1.5E+07	1.0E+08	1.0E+07	1.2E+07
3	3.5E+05	6.4E+06	5.2E+07	1.0E+07	8.6E+06	3	4.6E+06	2.6E+07	5.8E+07	3.8E+06	6.6E+06
4	1.4E+06	2.0E+07	3.2E+07	6.6E+06	6.5E+06	4	9.4E+06	2.5E+07	4.7E+07	7.2E+06	7.3E+06
5	4.1E+05	9.9E+06	2.6E+07	5.2E+06	8.6E+06	5	1.5E+05	1.4E+07	4.4E+07	7.3E+06	7.6E+06
6	5.6E+06	5.1E+06	5.5E+07	4.5E+06	5.0E+06	6	5.6E+05	7.6E+06	7.9E+07	5.1E+06	4.5E+06
7	2.7E+06	9.0E+06	4.0E+07	1.1E+07	1.1E+07	7	7.0E+07	1.0E+07	8.0E+07	9.5E+06	8.4E+06
8	3.4E+06	1.0E+07	1.9E+07	5.0E+06	4.8E+06	8	5.8E+06	1.7E+07	8.6E+07	3.6E+06	4.2E+06
Av	2.1E+06	9.6E+06	3.3E+07	6.4E+06	6.6E+06	Av	1.9E+07	1.5E+07	6.5E+07	6.4E+06	7.1E+06
SD	1.9E+06	4.8E+06	1.5E+07	2.5E+06	2.4E+06	SD	3.0E+07	7.8E+06	2.7E+07	2.5E+06	2.5E+06

Assay C) Populations on stigma first inoculated with Ea8862

0 h Ea8862 x Eh1087			Ea8862 x EhΔAGA			12 h Ea8862 x Eh1087			Ea8862 x EhΔAGA		
	N	R	N	R	R + S		N	R	N	R	R + S
1	1.1E+08	1.8E+05	1.6E+07	5.5E+04	4.8E+04	1	5.7E+07	1.2E+05	4.7E+07	1.3E+05	2.5E+05
2	6.9E+06	2.8E+04	2.3E+07	1.8E+05	1.1E+05	2	2.0E+07	3.3E+05	3.3E+07	1.6E+05	2.0E+05
3	7.9E+06	6.0E+04	1.3E+07	1.2E+05	1.2E+05	3	5.9E+07	3.8E+05	4.7E+07	5.0E+05	6.1E+05
4	8.9E+06	7.5E+04	1.3E+07	9.3E+04	8.6E+04	4	2.0E+07	2.5E+05	7.4E+07	3.1E+05	1.1E+05
5	1.1E+07	9.8E+04	1.4E+07	8.6E+04	4.3E+04	5	4.6E+07	1.9E+05	5.9E+07	5.5E+05	4.6E+05
6	6.0E+07	7.4E+04	4.6E+06	3.5E+05	2.3E+05	6	6.2E+07	2.7E+05	4.4E+07	4.0E+05	4.7E+05
7	8.8E+07	6.8E+04	1.4E+07	6.6E+04	5.0E+04	7	3.1E+07	2.0E+05	6.3E+07	3.5E+05	3.0E+05
8	4.3E+07	5.7E+04	5.5E+06	9.7E+04	1.2E+05	8	5.2E+07	1.5E+05	2.8E+07	1.5E+06	1.5E+06
Av	4.2E+07	8.0E+04	1.3E+07	1.3E+05	1.0E+05	Av	4.3E+07	2.4E+05	4.9E+07	4.9E+05	4.9E+05
SD	4.1E+07	4.5E+04	5.8E+06	9.7E+04	6.1E+04	SD	1.7E+07	9.0E+04	1.5E+07	4.4E+05	4.4E+05

24 h Ea8862 x Eh1087			Ea8862 x EhΔAGA			48 h Ea8862 x Eh1087			Ea8862 x EhΔAGA		
	N	R	N	R	R + S		N	R	N	R	R + S
1	6.6E+07	1.3E+06	4.9E+07	9.1E+05	7.4E+05	1	2.1E+08	1.5E+06	6.7E+07	2.3E+06	1.5E+06
2	6.9E+07	2.0E+05	1.1E+08	1.3E+06	1.2E+06	2	1.4E+08	9.4E+05	7.5E+07	4.0E+06	3.2E+06
3	1.2E+08	4.4E+06	7.5E+07	1.8E+05	1.3E+05	3	6.9E+07	3.1E+06	7.9E+07	2.4E+06	1.8E+06
4	6.5E+07	5.4E+05	7.2E+07	8.5E+05	6.3E+05	4	7.9E+07	1.5E+06	6.0E+07	1.2E+06	8.1E+05
5	6.1E+07	2.9E+05	6.8E+07	1.7E+05	1.2E+05	5	1.2E+08	3.8E+06	2.0E+08	2.4E+06	3.9E+05
6	9.5E+07	4.4E+05	6.8E+07	1.2E+06	7.0E+05	6	1.2E+08	1.1E+06	2.8E+08	1.1E+06	2.8E+05
7	8.8E+07	2.1E+06	1.1E+08	2.7E+05	3.1E+05	7	1.2E+08	7.2E+06	3.2E+08	6.0E+06	8.6E+05
8	4.0E+07	3.9E+05	6.6E+07	4.6E+06	3.2E+06	8	7.1E+07	1.1E+06	6.9E+08	5.0E+06	9.0E+05
Av	7.5E+07	1.2E+06	7.8E+07	1.2E+06	8.7E+05	Av	1.2E+08	2.5E+06	2.2E+08	3.0E+06	1.2E+06
SD	2.4E+07	1.4E+06	2.2E+07	1.5E+06	1.0E+06	SD	4.7E+07	2.2E+06	2.2E+08	1.8E+06	9.5E+05

72 h Ea8862 x Eh1087			Ea8862 x EhΔAGA		
	N	R	N	R	R + S
1	4.8E+07	7.7E+06	9.7E+07	5.0E+06	4.3E+06
2	5.0E+07	7.4E+06	9.9E+07	3.6E+06	2.8E+06
3	1.7E+08	2.6E+06	1.5E+08	9.5E+06	6.8E+06
4	1.5E+08	3.2E+06	1.4E+08	5.9E+06	5.5E+06
5	3.2E+08	5.2E+05	4.4E+07	4.1E+06	4.4E+06
6	1.2E+08	3.2E+06	4.8E+07	4.4E+06	2.7E+06
7	7.7E+07	5.5E+06	1.9E+08	4.8E+06	4.7E+06
8	1.7E+08	1.7E+06	1.1E+08	4.5E+06	3.6E+06
Av	1.4E+08	4.0E+06	1.1E+08	5.2E+06	4.4E+06
SD	8.9E+07	2.6E+06	4.9E+07	1.9E+06	1.4E+06